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IDENTIFICATION OF GENES CONTROLLING THE ACTION OF 9-LOX OXYLIPINS IN PLANT DEFENSE

Ph.D. Thesis

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IDENTIFICATION OF GENES CONTROLLING THE ACTION OF 9-LOX OXYLIPINS IN PLANT DEFENSE

BY

SATISH KULASEKARAN

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DECLARATION

I hereby declare that the dissertation entitled **"IDENTIFICATION OF GENES CONTROLLING THE ACTION OF 9-LOX OXYLIPINS IN PLANT DEFENSE"** submitted to the Departamento de Biología Molecular, Facultad de Ciencias, Universidad Autónoma de Madrid is a record of research work done by me, under the supervision of Prof. Carmen Castresana Fernández at the Department of Plant Molecular Genetics, CNB-CSIC, Madrid and that, to the best of my knowledge and belief, it neither contains material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning.

Satish Kulasekaran

(Doctoral candidate)

To my dad, mom and brother

And to my Papa

அறிவுடையார் எல்லா முடையார் அறிவிலார்

என்னுடைய ரேனும் இலர்.

- திருவள்ளுவர் (நாற்பதாம் திருவள்ளுவர் ஆண்டு)

Those who possess wisdom possess everything; those who have not wisdom, whatever they may possess, have nothing.

- Thiruvalluvar (1 A.D.)

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Abbreviations

ABBREVIATIONS

$^1O_2$	singlet oxygen
9-HOT	9-hydroxy octadecatrienoic acid
9-KOT	9-ketooctadecatrienoic acid
aa	amino acid
ABA	abscisic acid
ATP	adenosine triphosphate
avr	avirulence gene
bp	base pair
$CaCl_2$	calcium chloride
CAPS	Cleaved Amplified Polymorphic Sequences
cDNA	complementary DNA
cfu	colony forming units
Col-0	Columbia
COR	coronatine
DAB	3,3'- diaminobenzidine
DAF-2DA	4,5-diaminofluorescein diacetate
DNA	deoxyribonucleic acid
ETI	effector triggered immunity
GCN	General Control of Nonderepressible
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HEAT	Huntington's, Elongation factor EF3, protein phosphatase2A and yeast PI3-kinase TOR1
HR	hypersensitive reaction
Ila	Ilityhia
JA	jasmonic acid
KB	King's B medium
Kb	kilo base
KCl	potassium chloride
LB	Luria Bertani

Abbreviations

L*er*	Landsberg *erecta*
LOX	lipoxygenases
MASC	Max-Planck Arabidopsis SNP Consortium
MES	morpholino ethane sulphonic acid
MS	Murashige and Skoog
nm	nanometer
NO	nitric oxide
noxy	non responding to oxylipin
O_2^-	superoxide
O.D	optical density
PAMP	pathogen associated molecular pattern
PCR	Polymerase Chain Reaction
Pst	*Pseudomonas syringae* pathovar tomato
PTI	PAMP's triggered immunity
PUFA	polyunsaturated fatty acid
RB	Rose Bengal
RNA	ribonucleic acid
ROS	Reactive Oxygen Species
RT-PCR	reverse transcriptase-PCR
SA	salicylic acid
SNP	single nucleotide polymorphism
SSLP	simple sequence length polymorphism
T-DNA	transfer DNA
TTSE	type three secretion effector
TTSS	type three secretion system

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Abstract

ABSTRACT

Oxylipins are a class of lipid metabolites which play an important role in plant development and defense responses against pathogen attack. Principally they are synthesized by the oxygenation of fatty acid molecules through the action of enzymes like 9- and 13-lipoxygenases and α-dioxygenases or by chemical oxygenation. The 9-LOX oxylipin pathway is a nascent field of research and its role in the defense against pathogenic bacteria has been recently demonstrated. In this study, we carry out a genetic analysis to identify the signaling components of the 9-LOX pathway and to annotate its importance in plant defense responses. Through a forward genetic screen, three mutants, namely *noxy3*, *noxy72* and *noxy76*, from a population of randomly mutagenized *Arabidopsis thaliana* lines were identified to be insensitive to treatment with the 9-lipoxygense derivative, 9-hydroxy octadecatrienoic acid (9-HOT). Characterization of responses of these *noxy* mutants to the hemibiotrophs, *Pseudomonas syringae* has enabled us to identify two negative regulators of plant defense in *NOXY3* and *NOXY72*, as the mutations in these genes led to enhanced resistance in the stomata and apoplast, respectively. Contrarily, *noxy76* mutant exhibited susceptibility to *Pseudomonas,* with a stronger defect in the pre-invasive defense. Mapping of these mutations have revealed that *noxy3* and *noxy72* encode mitochondrial proteins, namely LON1 protease and a S-adenosyl methionine dependent methyl transferase, respectively. In addition to this, *noxy76* was mapped to a HEAT repeat protein called ILITYHIA. Further characterization revealed that *noxy76* was impaired in stomatal closure in response to bacteria and Reactive Electrophilic Species-Oxylipins, whereas this defect was independent of ABA. Moreover, characterization of responses to ROS and NO revealed that *noxy76* was impaired in signaling singlet oxygen and NO and that it was deficient in the production of NO in the stomata. These results when taken together indicated that the defect of *noxy76* in producing and signaling NO resulted in the failure of activating defense against bacteria.

Introduction

1. INTRODUCTION

1.1. Plant-Pathogen interactions

Plants are sessile organisms and are constantly threatened by a myriad of pathogenic organisms like bacteria, virus, fungi and herbivorous pests. They can mount a strong innate immune response on the invading pathogen so as to limit the damage it incurs on the host cell. Plant pathogens establish a parasitic interaction with the host plants and based on their lifestyle they can be fundamentally, divided into two groups, namely biotrophs and necrotrophs. Biotrophs derive nutrients from the living host tissue through specialized structures like haustoria, and the obligate oomycete pathogen, *Peronospora parasitica* is a classic example of this group (Koch and Slusarenko, 1990). The necrotrophs kill the host cells and derive nutrition from those dead cells. The fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* belong to the necrotrophic group of pathogen which can cause extensive damage to the host tissue. Some plant pathogens like *Pseudomonas syringae* display both lifestyles and are called as hemibiotrophs. *Pseudomonas* infects plants through wounds and stomata and multiplies in the intercellular spaces. In the early stages of infections, host cell death does not occur, but later stages of infection are associated with host tissue chlorosis and necrosis (Glazebrook, 2005).

The perception of pathogens by the plant can lead to the activation of defense responses which ultimately results in either an incompatible interaction in which the plant becomes resistant and mounts a defense response controlling the invading pathogen, or a compatible interaction in which the host becomes susceptible due to the failure in activating a defense response.

1.2. Plant defense responses

Plants rely on innate immune responses for protection against pathogens and two layers of innate immune responses have been identified. One uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs), such as flagellin and is called PAMP's or MAMP's triggered immunity (PTI). The second acts largely inside the cell, using the polymorphic NB-LRR protein products encoded by most R genes, in which an effector molecule from the pathogen is specifically recognized by one of the nucleotide binding (NB) and Leucine Rich Repeats (LRR domains) (NB-

LRR) proteins, resulting in effector-triggered immunity (ETI), which is effective against biotrophic and hemibiotrophic pathogens but ineffective against necrotrophs (Jones and Dangl, 2006).

1.2.1. Pathogen- or Microbes- Associated Molecular Patterns (PAMP's or MAMP's) Triggered immunity (PTI)

The perception of pathogenic epitopes by the transmembrane Pattern Recognition Receptors (PRR's) becomes the first and the foremost aspect of plant defense and leads to an unspecific immune response called as PAMP's triggered immunity (PTI) that provides the basal resistance to the plants. The best example of this type of immunity is the recognition of the bacterial peptide flagellin by the Leucine-Rich Repeat domains of the plant receptor protein, Flagellin Sensing 2 (FLS2), a transmembrane receptor like kinase (Gomez-Gomez and Boller, 2000) whose perception of flagellin leads to the activation of the plethora of defense responses.

1.2.2. Effector triggered immunity (ETI)

During co-evolution of host and pathogen, plants have developed another layer of defense based on the detection of effector proteins (Chisholm *et al*., 2006; Jones and Dangl, 2006). When the invading microorganism is able to overcome the basal resistance by suppressing the PTI of the plant, a secondary and more efficient resistance is initiated by plants, as a selection pressure is exerted in plants to evolve new mechanisms to evade pathogen virulence. This secondary resistance is called effector triggered immunity which occurs largely inside the cell and consists of activation of a certain set of resistance (R) genes (Jones and Dangl, 2006; Martin *et al*., 2003; Nimchuk *et al*., 2003). R proteins are polymorphic and a majority of them are characterized by the NB-LRR proteins (Dangl and Jones, 2001). These proteins recognize a wide variety of pathogen effectors and activate resistance mechanisms in plants. In the case of an incompatible reaction between the pathogen and the host, the recognition of effector molecules by the plant R proteins activate a robust defense response resulting in a hypersensitive reaction (HR) characterized by an apoptotic localized cell death which controls the spread of the pathogen and leads to plant resistance.

1.2.3. Immune responses triggered during PTI and ETI

Immune responses triggered by PRRs and R-gene-products are very similar (Navarro *et al*., 2004; Tsuda *et al*., 2009; Hammond-Kosack and Parker, 2003; Jones and Dangl, 2006). However, constitutive defense components and associated signaling events playing major roles in these two immunity barriers might differ (Zipfel *et al*., 2008; Thilmony *et al*., 2006; Truman *et al*., 2006; Navarro *et al*., 2004). Overall, these responses involve ion fluxes across the plasma membrane, the generation of reactive oxygen intermediates (ROI), nitric oxide (NO), deposition of callose, activation of calcium-dependent and mitogen-activated protein kinases (MAPK), and transcription of numerous defense genes.

(a) Changes in ion fluxes and increase in cytosolic Ca^{2+} levels

The direct implication of the interaction of MAMP's with the receptor leads to drastic changes in ion fluxes across the plasma membrane within 2 min of elicitation, notably of H^+, K^+, Cl^- and Ca^{2+} (Nurnberger *et al*., 2004; Garcia-Brugger *et al*., 2006; Pugin *et al*., 1997). Elevation of Ca^{2+} levels constitutes the signal for downstream events (Chandra *et al*., 1997). The abolition of MAPK phosphorylation by Ca^{2+} inhibitors further demonstrated the significance of Ca^{2+} in pathogen induced stress (Vadaserry *et al*., 2009).

(b) Production of Reactive Oxygen Species

One of the most rapid and robust response of plants to pathogen attack is the oxidative burst during which molecular oxygen can be converted to different ROS like superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$) and singlet oxygen($^1O_2$) through distinct reactions (Apel and Hirt, 2004, Jabs *et al*., 1997). The defense responses associated with the generation of ROS includes direct killing of pathogen, activation of host cell death and cell wall strengthening. ROS production during pathogen attack is initiated through enhanced enzymatic activity of plasma-membrane bound NADPH-oxidases, cell wall–bound peroxidases and amine oxidases in the apoplast (Grant and Loake, 2000). Thus during plant pathogen interaction, a suppression of the plant ROS detoxifying mechanisms becomes important for the accumulation of ROS and the onset of Programmed Cell Death (PCD) (Apel and Hirt, 2004). A biphasic generation of hydrogen peroxide occurs during an incompatible interaction leading to activation of PCD to restrict the pathogen, whereas during the compatible interactions, only a single peak of hydrogen peroxide is observed (Baker and Orlandi, 1995). Even though more research has been focused on

the role of hydrogen peroxide and superoxide in plant defense, the significance of singlet oxygen and hydroxyl radical is starting to unravel. Phenaleno-like phytoalexins and phytoanticipins might act as photosensitizers to generate $^1O_2$ following absorption of light energy (Flors and Nonell, 2006), indicating that increased production of these compounds can lead to the generation of $^1O_2$ during pathogen attack (op den Camp *et al*., 2003).

(c) Nitric oxide in plant defense responses

Nitric oxide (NO) is a highly bioreactive molecule which exerts number of diverse signaling functions in phlyogenetically distant species, and its cytotoxic and cytoprotecting roles have been described (Beligini and Lamattina, 2001; Beligni *et al*., 2002, Bethke *et al*., 2006, Crawford and Guo, 2005). During the hypersensitive reaction to an avirulent pathogen, the generation of NO occurs and in conjunction with ROS it can activate hypersensitive cell death in soybean and tobacco (Delledone *et al*., 1998; de Pinto *et al*., 2002). NO also plays a role in elevating free cytosolic Ca^{2+} in tobacco cells that are responding to hyperosmotic stress or cryptogein and along with cGMP and cADPR, mediates ABA induced stomatal closure in guard cells (Lamotte *et al*., 2004; Neill *et al*., 2002; Garcia-Mata and Lamattina, 2001). NO also shares a cross-talk with salicylic acid (SA) and jasmonic acid (JA) signaling. Treatment of tobacco leaves with NO increased the endogenous levels of SA and nitric oxide synthase (NOS) inhibitors and NO scavengers attenuated SA-induced SAR (Durner *et al*., 1998; Song and Goodman, 2001).

(d) Callose and lignin deposition

During the early stages of pathogen attack, plants are able to induce the formation of physical barriers called papillae which are cell wall appositions whose main constituent is callose, an amorphous high molecular weight β-(1,3)-glucan polymer that serves as a matrix in which anti-microbial compounds can be deposited for delivery of chemical compounds at the site of pathogen attack (Brown *et al*., 1998). *Arabidopsis* cotyledons have been shown to induce callose formation upon treatment with the peptide flg22, derived from flagellin (Luna *et al*., 2011).

Lignin is a phenolic polymer resulting from the oxidative polymerization of monolignols and is a major component of secondary cell wall (Boerjan *et al*., 2003). Lignin along with suberin is the constituent of the wound periderm which acts as a mechanical barrier during plant pathogen interaction and plants with higher lignin content are more resistant to microbes (Schreiber *et al*., 1999; Kawasaki *et al*., 2006).

(e) Activation of MAP Kinases

flg22 is known to trigger a rapid and strong activation of MAPK3, MAPK4 and MAPK6 (Droillard *et al*., 2004). The MAP kinase cascade includes a series of phosphorylation events which lead to the activation of subsequent MAP kinases. MAPK3 and MAPK6 are essential for the activation of the camalexin biosynthetic pathway which is an important phytoalexin, whose absence leads to increased susceptibility to *Botrytis cinerea*. This susceptibility was similar to the susceptibility of the *pad4* mutant which is also necessary for camalexin biosynthesis (Ferrari *et al*., 2007).

1.2.4. Hormonal crosstalk during plant pathogen interactions

Phytohormones are small molecules which play a crucial role in plant growth development, reproduction and survival and changes in hormone concentration can be triggered under biotic and abiotic stress conditions. Studies using mutants and transgenic lines impaired in hormone biosynthesis, signaling or perception have revealed a severe alteration in the level of resistance against specific pathogens (Pieterse *et al*., 2009). The significance of the phytohormones, SA, JA and Ethylene in regulating plant defense responses against various pathogens, pests and wounding has been well established (Glazebrook 2005; del Pozo *et al*., 2004; van Loon *et al*., 2006; Loake *et al*., 2007). Recent studies have also highlighted the importance of Abscisic acid (ABA), auxins, gibberellins and brassinosteroids in plant defense responses (Asselbergh *et al*., 2008; Wang *et al*., 2007; Navarro *et al*., 2008; Nakashita *et al*., 2003).

SA plays a significant role in the activation of defense responses against biotrophic and hemibiotrophic plant pathogens as well as the establishment of long lasting and broad spectrum induced resistance called systemic acquired resistance (SAR) and is characterized by an activation of a set of pathogenesis related (PR) genes which encode proteins with antimicrobial activity (van Loon *et al*., 2006). SAR activation was impaired in mutants of SA synthesis and signaling thereby highlighting the importance of SA in SAR (Durrant and Dong 2004). SA mediated changes in the cellular redox potential activates the regulatory protein NPR1 (Non-expressor of PR1 genes) which in turn acts as a transcriptional co-activator of SA-responsive genes (Loake *et al*., 2007; Dong 2004).

JA and ET, on the contrary, play an important role in defense responses against necrotrophic pathogens and herbivorous insects and operate synergistically to activate expression of defense related genes after pathogen attack (Penninckx *et al*., 1996; Thomma *et al*., 2001). *Arabidopsis* mutants deficient in JA biosynthesis or signaling are highly susceptible to insect attack

and induction of JA synthesis was observed during wounding by herbivore feeding. JA also plays a role in Induced Systemic Resistance (ISR), wherein JA synthesized in response to cell damaged is transported systemically to activate responses in distal tissues and hence one type of stress might lead to cross-protection against others (Browse, 2009). ISR was shown to be effective against pathogens and insects that are sensitive to JA and ET dependent defense responses (van Oosten *et al*., 2008). Furthermore, the *Arabidospsis* transcription factor, Ethylene Response Factor (ERF) acts as a positive regulator of JA and ET signaling and members of the ERF family have been shown to play important role in mediating plant defense responses (McGrath *et al*., 2005).

It is becoming increasingly evident that there exists a complex crosstalk between these hormones (Bari and Jones, 2009). Antagonistic interactions have been revealed between JA and SA in many studies. Application of JA depressed the SA dependent PR genes activation and the activation of expression of JA-dependent PDF gene in response to *Alternaria brassicicola* was higher in SA-deficient NahG transgenic plants (Niki *et al*., 1998; Penninckx *et al*., 1996). On the other hand, SA-dependent PR gene expression was activated to higher levels during enhanced resistance to *Pseudomonas syringae* in mutants compromised in JA signaling (Kachroo *et al*., 2001). A synergistic interaction occurs between JA and ET which was well documented in the regulation of the *PDF* gene which requires concomitant activation of JA and ET pathways (Penninckx *et al*., 1996) and the members of ERF family were identified as integrators of JA and ET pathway (Pre *et al*., 2008).

Recent studies have indicated a bidirectional role for ABA in plant immunity. ABA plays a crucial role in pre-invasive defense against microbial pathogen as it is an important constituent of the stomatal closure mechanism thereby enabling resistance (Melotto *et al*., 2006). On the other hand, during post-invasive defense responses, ABA insensitive mutants exhibited enhanced resistance while ABA hypersensitive mutants remained susceptible to *Pseudomonas syringae* (de Torres-Zabala *et al*., 2007; Goritschnig *et al*., 2008, Cao *et al*., 2011, Fan *et al*., 2009).

Plants thus modulate the levels of each one of the phytohormones to modify defense-related genes and coordinate complex interactions to activate an effective defense response against attack by different pathogens (Robert-Seilaniantz *et al*., 2011).

1.3. *Arabidopsis* - *Pseudomonas syringae* system of plant pathogen interaction

The model host-pathogen interaction system of *Arabidopsis-Pseudomonas syringae* has enabled the functional characterization of innate immunity, susceptibility, and gene-for-gene resistance and has been used for transcriptomics and proteomics studies of these responses (Jones *et al*., 2004). *Pseudomonas syringae* is a Gram negative rod shaped bacterium and the different strains of this bacterium are known to interact in a host-specific manner (Hirano and Upper, 2000). In nature *Pseudomonas syringae* exhibits an epiphytic growth, i.e., surviving and/or multiplying on plant surface, before entering the plant predominantly through wounds or natural openings like stomata or hydathodes (Katagiri *et al*., 2002). Once it gains entry into the intercellular spaces, it multiplies rapidly upon successful colonization with the help of type III secretion system and develops disease symptoms which are characterized by necrotic lesions surrounded by chlorotic tissue.

Arabidopsis has evolved two distinct layers of defense in response to *Pseudomonas syringae* pathovars tomato (*Pst* DC3000). The first layer of defense is concerned with the entry level defense, the so called stomatal defense and the second layer is responsible for the defense responses at the intercellular level which is called as apoplastic defense.

The limited amount of nutrients on the surface of the plant restricts the growth and development of the *Pseudomonas syringae* and hence it becomes important for *Pseudomonas syringae* to enter into the apoplastic space to establish an endophytic phase for rapid multiplication and to develop disease symptoms (Hirano and Upper, 2000). As *Pseudomonas syringae* lacks cell wall degrading enzymes to bore through the leaf surface, it has to enter the host through natural openings in the plant. Out of the various natural openings present in plants, stomata outnumber the rest of the openings and hence are the predominant entry points for *Pseudomonas syringae*. The notion that stomata are passive ports for entry of pathogen into the apoplastic space was proven wrong by the pioneering work of Melotto *et al*., (2006), in which an unexpected function for stomata was revealed.

Stomata act as innate immunity gates to prevent the entry of pathogen into the apoplastic space. *Arabidopsis* was able to close its stomata in presence of live bacteria, flg22 and lipopolysaccharide and this mechanism requires PAMP signaling and homeostasis of the defense hormone SA, and hence forms an important part of plants innate immunity. The *Arabidopsis*

receptor protein FLS2 which plays an important role during PTI also has a significant role in stomata mediated immunity, as stomata of *fls2* mutants did not sense the presence of bacteria and remains open. In addition to it, stomata of ABA biosysnthetic (*aba 3-1*) and signaling mutants (*ost1-2*) and SA deficient mutants (*nahG* and *eds16*) also did not respond to the presence of PAMP's on the surface. Furthermore, in response to *Pst* DC3000, the stomata close within 1hr of perception and reopened after 3hrs, indicating that *Pst* DC3000 has evolved a mechanism to inhibit the stomatal closure response of *Arabidopsis*. This result indicated that plants have evolved a mechanism to control the entry of pathogens through the stomata thereby reducing the amount of bacteria that enter and hence play an active role in plant innate immunity (Melotto *et al*., 2006).

The ion channel regulation forms an integral part of the light mediated stomatal opening and the bacterial flagellin is known to inhibit light-induced stomatal opening by inhibiting the K^+ channels of guard cells that mediate K^+ uptake during stomatal opening and coronatine produced by *Pst* DC3000 reverses this inhibitory effect indicating interplay between plant and pathogen in the regulation of ion channels (Zhang *et al*., 2008).

The apoplastic space of the plant serves as a potential niche for *Pseudomonas syringae* to exploit the nutrients of the plant and develop symptoms. Two virulence factors are known to play an important role for development of the disease symptoms, namely the Coronatine and Type III Secretion System (TTSS).

Coronatine (COR) is a non-host specific polyketide phytotoxin produced by various pathovars of *Pseudomonas syringae* and has been implicated in the induction of chlorosis in the host tissue. COR shares remarkable structural similarity with methyl jasmonate and serves as a potent inducer of jasmonate responsive genes and thereby increases the pathogenicity of *Pst* DC3000 (Yan *et al*., 2009; Uppalapatti *et al*., 2007) in conjunction with JA, and COR is also known to inhibit callose deposition in leaf mesophyll cells and root cells (Clay *et al*., 2009; Millet *et al*., 2010). Besides the apoplastic activities of coronatine, it reopens the stomata of *Arabidopsis* after 3 hrs of inoculation leading to increased bacterial entry and colonization thereby enhancing pathogenicity of *Pst* DC3000 by suppressing stomatal defense responses. Meanwhile, *Pst* DC3000 deficient in the production of coronatine (*Pst* DC3118) was unable to reopen the stomata of *Arabidopsis* after 3 hrs of inoculation which highlighted the importance of coronatine in the pathogenicity of *Pst* DC3000 (Melotto *et al*., 2006). Furthermore, the growth and development of symptoms by coronatine deficient *Pst* DC3000 was reduced in comparison to *Pst* DC3000 upon surface inoculation indicating (Mittal and Davis, 1995).

The *hrp* (hypersensitive response and pathogenicity) gene cluster and *hrc* (HR and conserved) genes encode the type III secretion system and avirulence (*avr*) genes and Hrp-dependent outer protein (hop) genes encode the effector proteins (Alfano and Collmer, 1997). The whole repertoire of genes encoding TTSS proteins have been identified through bioinformatics and functional screens (Cunnac *et al*., 2009). The transmission of effectors occurs via the *Pseudomonas syringae* pilus which is assembled by the TTSS and acts as a bridge for transferring the effectors into the host tissue (Roine *et al*., 1997; Wei *et al*., 2000; Hu *et al*., 2001).

Defense responses in *Arabidopsis* are induced by the recognition of flg22 by the FLS2 receptor via the activation of MAP kinases, MPK3 and MPK6 (Asai *et al*., 2002). The first target of the effectors of *Pseudomonas* is to suppress the PTI to enable an infection of the host. The Hop effectors from the Type III secretion effector (TTSE) of *Pseudomonas* display a phosphothreonine lyase activity which inactivates the MAP kinases, thereby suppressing downstream events leading to immunity in the host (Zhang *et al*., 2007). Furthermore, *Pseudomonas* strains unable to deliver effectors into plant cells, such as *Pst* DC3000 *hrpA*$^{-}$ (*hrpA*$^{-}$), cannot establish an infection. In this case, basal resistance of the host is sufficient to restrict pathogen growth (Roine *et al*., 1997). In contrast, treating plants with the virulent strain *Pst* DC3000 with a full TTSS leads to a successful infection because basal resistance of the host is overcome TTSE of the pathogen. Moreover, *Pseudomonas syringae* being an extra cellular pathogen manipulates the host secretion system to promote successful invasion of plants (Kaffarnik *et al*., 2009).

Once *Pseudomonas syringae* starts to pump effector molecules into the apoplast through the TTSS, two kinds of reaction can occur; if an infected *Arabidopsis* plant has an *R* gene that recognizes a *P. syringae* type III effector (i.e., an Avr protein in this situation), a rapid defense mechanism of the plant will be triggered. Alternatively, if the infected *Arabidopsis* plant has no corresponding *R* gene and/or the *P. syringae* strain has no *avr* gene, defense responses will be activated slowly, the infection will continue, and the plant will succumb to *P. syringae* and become diseased. Besides the activation of R genes, a plethora of responses are initiated inside the cell which include generation of reactive oxygen intermediates, activation of hormone signaling pathways and activation of cell death pathways in infected cells so as to control the growth of bacteria. The most important of them is the induction of the SA-dependent signaling pathway. The activation of the SA pathway is frequently accompanied by a cell death reaction at the infection zone (hypersensitive response, HR) that limits the access of the bacteria to nutrient pools (Hammond-Kosack and Parker, 2003; Jones and Dangl, 2006; Boller and Felix, 2009).

1.4. Oxylipins

One of the important mechanisms by which plants respond to the presence of pathogen is the generation of oxygenated lipid derivatives and further transformation of fatty acids, leading to the formation of a group of metabolites, collectively called as oxylipins. These compounds are similar in many ways to the eicosanoids derived from arachidonate in animals, which have so many varied functions but especially in the inflammatory process. Fatty acids serve as an important source of energy and also form part of complex lipids which are the constituents of cellular membranes. Fatty acids and their derivatives act as signaling molecules and modulate a variety of responses to biotic and abiotic stresses which include tolerance to temperature, salt drought and heavy metal stress (Iba, 2002; Routaboul *et al*., 2000; Upchurch *et al*., 2008). The FA-derived phytohormone, jasmonic acid is particularly well known for its role in wound responses and plant defense against insect pathogens (Creelman and Mulpuri, 2002). Moreover jasmonic acid and its immediate precursor 12-oxo-phytodienoic acid (OPDA) act as signaling molecules to induce the expression of a distinct set of genes (Block *et al*., 2005).

1.4.1. Biosynthesis of Oxylipins

The formation of oxylipins is one of the main reactions in lipid alteration and it starts with the release of fatty acids from the membranes by the action of lipases. The initial formation of hydroperoxides can occur in 3 distinct ways; (i) by auto oxidation, (ii) enzymatically by the action of lipoxygenases, α-dioxygenases or the endoplasmic reticulum localized cytochrome P450 enzymes and (iii) non-enzymatically in the presence of singlet oxygen or free radical catalyzed mechanisms. The oxidation is followed by secondary modifications which are catalyzed by other enzymatic activities. In plants, oxylipin formation occurs predominantly from linoleic acid and α-linolenic acid (Spiteller *et al*., 2001; Feussner *et al*., 2001; Mueller and Berger, 2009; La Camera *et al*., 2004).

Preferentially, oxidation of fatty acids occurs by the enzymatic route through the action of lipoxygenases and α-dioxygenases (Hamberg *et al*., 1999; Feussner and Wasternack, 2002). Lipoxygenases are non-heme iron containing dioxygenases which catalyze the incorporation of molecular oxygen in polyunsaturated fatty acids (PUFA) forming hydroperoxy fatty acids. Plant lipoxygenases are classified into two types based on the position in which they oxygenate linoleic acid, namely, 9-lipoxygenase and 13-lipoxygensae which incorporate molecular oxygen at carbon

positions 9- and 13- of the hydrocarbon backbone of the fatty acid. This oxygenation process leads to two corresponding groups of compounds, 9-hydroperoxy and 13-hydroperoxy derivatives of Linoleic acid (Liavonchanka and Feussner, 2006). In *Arabidopsis thaliana*, six lipoxygenases have been identified (*At*LOX1 to *At*LOX6), which are position specific. The LOXes, *At*LOX1 and *At*LOX5 are 9-lipoxygenases and *At*LOX2, *At*LOX3, *At*LOX4 and *At*LOX6 are 13-lipoxygenases (Bannenberg *et al*., 2009).

The 9- and 13- hydroperoxy derivatives of PUFA are subsequently metabolized through a number of secondary reactions which include the following metabolic pathways:

- Peroxigenase (POX) pathway: Intramolecular oxygen transfer converts fatty acid hydroperoxides to epoxy- or dihydrodiol polyenoic fatty acids.
- AOS pathway: Allene oxide synthase (AOS) converts hydroperoxides to octadecanoids which are later converted to jasmonates.
- HPL pathway: Hydroperoxide lyase catalyzes the cleavage of the hydrocarbon backbone of fatty acid hydroperoxides leading to formation of short chain aldehydes and their corresponding C12 or C9 ω-fatty acids.
- DES pathway: The action of divenyl ether synthase (DES) leads to the formation of colneleic acid and colnelenic acid.
- LOX catalyzed hemolytic cleavage leading to the formation of ketodienes.
- EAS pathway: Epoxy hydroxy fatty acids are formed by intramolecular rearrangement of hydroperoxy fatty acids catalyzed by epoxy alcohol synthase (EAS).
- Reductase pathway: hydroperoxy fatty acids are reduced to their corresponding hydroxy derivatives and this reaction can be facilitated by glutathione.

α-dioxygenases catalyze the oxygenation of fatty acids at the C-2 position of the hydrocarbon backbone leading to the formation of 2-hydroperoxy derivative. α-dioxygenases show high homology towards both mammalian prostaglandin-synthase isoforms (PGHS-1 and PGHS-2) and both enzymes share common catalytic features (Koszelak-Rosenblum *et al*., 2008). The resulting compound is very unstable and decomposes non-enzymatically into CO_2 and the corresponding chain shortened fatty aldehyde. Alternatively the hydroperoxy fatty acid may be reduced to corresponding 2-hydroxy fatty acid (Hamberg *et al*., 2002).

Oxylipins that have been identified which are indicative of non-enzymatic lipid peroxidation include 10-hydroxy octadecadienoic acid (10-HOD), 12-HOD, 10-hydroxy octadecatrienoic acid (10-

HOT) and 15-HOT. These are specifically formed by the oxygenation reaction of linoleic or α-linolenic acid with $^1O_2$ (Durand *et al.,* 2009).

The oxylipin metabolism largely depends on the type of species, the nature of encountered stress and the location of the affected tissue. Fatty acid precursor availability is an essential determinant of the flux of compounds that are synthesized (La Camara *et al*., 2004).

1.4.2. Functions of Oxylipins

Oxylipins are involved in a number of physiological processes which include plant growth and fertility, mechanotransduction, adaptation to adverse growth conditions and in defense reactions after the attack of a pathogen (Sanders *et al*., 2000; Stintzi and Browse, 2000; Staswick *et al*., 2002; Blée, 2002). Numerous studies aimed at defining the action of oxylipins have shown that the expression of genes encoding the enzymes initiating the synthesis of oxylipins is specifically induced upon inoculation with plant pathogens (Sanz *et al*., 1998; Jalloul *et al*., 2002; Turner *et al*., 2002). Moreover, alterations in the synthesis of oxylipins in mutants and transgenic lines have been shown to modify the plant response to pathogen infection (Rance *et al*., 1998; De Leon *et al*., 2002; Farmer *et al*., 2003).

The best-characterized oxylipins are JA and its immediate precursor 12-oxo-phytodienoic acid (OPDA), which are formed enzymatically and accumulate in response to various stresses, in particular wounding and pathogen infection (Block *et al*., 2005). JA and OPDA have been identified as signaling molecules and their signaling leads to the interaction of the F-box ubiquitin ligase CORONATINE-INSENSITIVE1 (COI1) with JAZ transcriptional repressors, mediating degradation of these repressors of downstream JA-induced genes, many of which are dependent on the key transcription factor MYC2/JIN1 (Fonseca *et al*., 2009). JA is involved in regulating development processes which include growth inhibition, senescence, tendril coiling, flower development, tuber formation and leaf abscission. Furthermore, wounding response is one of the most characterized responses of the Jasmonate pathway during which an increased expression of JA biosynthetic enzymes occur leading to expression of proteinase inhibitors and foliar compounds which ultimately inhibit herbivory (Wasternack, 2007). JA is the key defense hormone that interacts with additional pathways to control resistance against necrotrophic pathogens (Glazebrook, 2005; Browse, 2009). Other 13-LOX derivatives like OPDA, 13-hydroxy-octadecatrienoic acid and 13-hydroperoxide lyase derived aldehydes are also regulators of plant defense gene expression (Vollenweider *et al*., 2000; Stintzi *et al*., 2001; Montillet *et al*., 2005; Prost *et al*., 2005).

α-dioxygenases have been implicated in plant defense and development. In tobacco and *Arabidopsis*, α-dioxygenases play an important role in defense against microbial pathogens during which their expression levels increase to higher levels upon the onset of hypersensitive reaction (Hamberg *et al*., 2003; De Leon *et al*., 2002). In addition, this enzymatic activity plays a role in the development of tomato and Nicotiana plants (Obregon *et al*., 2001; Bannenberg *et al*., 2009; Steppuhn *et al*., 2010). Recent study with the mutant in one of the α-dioxygenases, *dox1*, indicated that during *Pst* DC3000 inoculation, the absence of α-DOX activity leads to reduced activation of SAR (Vicente *et al*., 2012).

The oxylipins derived from the 9-LOX pathway are involved in plant defense responses in tobacco and *Arabidopsis* (Rance *et al*., 1998; Vellosillo *et al*., 2007; Hwang and Hwang; 2010; López *et al*., 2011). One of the 9-LOX oxylipin, 9-hydroxy octadecatrienoic acid (9-HOT) is known to induce root waving phenotype and inhibit the formation of lateral roots and the LOXes 1, 3 and 5 are expressed in lateral root primordial thereby playing a role in lateral root development. Furthermore, mutations in *LOX1* and *LOX5* increase the number of lateral roots and also susceptibility to bacterial pathogen. 9-HOT was able to induce oxidative stress in roots of wild type plants accompanied by callose deposition and activation of defense genes which indicated that the 9-LOX pathway function in cell wall modifications required for pathogen arrest (Vellosillo *et al*., 2007). In addition to this, 9-lipoxygenase derived oxylipins antagonize with ethylene in the control of oxidative stress, lipid peroxidation and plant defense (López *et al*., 2011). Besides this, *LOX5* encoded 9-LOX derived oxylipins function as susceptibility factor in plant interaction with green peach aphid wherein *LOX5* facilitates insect feeding from sieve elements and water consumption from xylem (Nalam *et al*., 2012). Further studies have revealed the metabolic interaction between the 9-LOX and α-DOX1 oxylipin pathways, and that the coordinated action of both enzymatic activities is required to activate local and systemic defense against *Pst* DC3000 in *Arabidopsis* (Hamberg *et al*., 2003; Vellosillo *et al*., 2007; Vicente *et al*., 2012). Additional support for the role of 9-LOX and α-DOX1 in plant defense comes from studies showing the action of specific oxylipins as defensive compounds reducing bacterial growth and symptoms, and signaling defense responses through a JA-independent pathway (Hamberg *et al*., 2003; Vicente *et al*., 2012; Vellosillo *et al*., 2007). In these studies, 9-ketooctdecatrienoic acid (9-KOT), the 9-LOX derivative showing the strongest protecting effect, was found to contribute to plant defense by interfering with the hormonal changes caused by bacterial effectors, preferentially the production of ABA. In accordance with these results, *lox1 dox1* seedlings (deficient in 9-KOT production) were proven to be hypersensitive to the growth inhibitory effect of ABA and showed enhanced activation of ABA-

inducible marker genes. Thus, indicating the action of 9-LOX and α-DOX as modulators of ABA homeostasis.

1.4.2.1. Oxylipins in stomata mediated immunity

By analysis of cell type-specific leaf transcriptome, *LOX1* was identified as a 9-specific LOX encoding gene of *Arabidopsis* whose expression is observed in guard cells (Leonhardt *et al*., 2004). Recent unpublished data have highlighted the importance of LOX1 activity in pre-invasive defense responses in *Arabidopsis*. In this work it has been revealed that LOX1 activity controls bacterial- and PAMP-induced stomatal closure and products of LOX activity such as fatty acid hydroperoxides and reactive electrophile oxylipins containing α, β-unsaturated structure induced stomatal closure (Montillet *et al*., unpublished) and thereby playing an important role in plant defense responses.

Despite mounting experimental evidences the mode of action of the oxylipins from the 9-lipoxygenase pathway is yet to be elucidated completely. In the present study we set out to characterize the genes which play a role in this pathway through the use of mutants impaired in their response to the 9-LOX derivative, 9-hydroxyoctadecatrienoic acid and evaluating their responses to pathogen inoculation.

Objectives

2. OBJECTIVES

The main objective of this work was to identify new components of the 9-LOX signaling pathway and to study their contribution in the defense response of plant to the infection of hemibiotrophic bacteria. To accomplish this, the following approaches were utilized:

1) Isolation and phenotypic characterization of mutants impaired in signaling responses to the 9-LOX oxylipin, 9-hydroxy octadecatrienoic acid (9-HOT).
2) Map-based positional cloning of selected mutants.
3) Examination of *noxy76*, a 9-HOT insensitive mutant failing to activate pre-invasive and apoplastic defenses and, thus showing enhanced susceptibility to the distinct *Pseudomonas* strains investigated in this study.

Materials & methods

3. MATERIALS AND METHODS

3.1. Plant material

The model plant utilized throughout this study was *Arabidopsis thaliana*, ecotypes Columbia (Col-0) and Landsberg *erecta* (L*er*).

T-DNA insertion mutant from NASC (Nottingham Arabidopsis Stock Centre) was obtained for *NOXY3* gene (SALK_013817).

3.2. Bacterial strains utilized

3.2.1. Bacterial phytopathogens

Psuedomonas syringae was the phytopathogenic bacteria utilized throughout this study and the three strains *Pseudomonas syringae pv. tomato* DC3000 *avrRpm1* (Debener *et al*.,1991; Innes *et al*.,1993), *Pseudomonas syringae pv. tomato* DC3000 (Whalen *et al*., 1991) and *Pseudomonas syringae pv. tomato* DC3000 *COR*$^{-}$ AK87 (Brooks *et al*., 2004) were selected for this study.

King's B (KB) medium containing 2% protease peptone, 2% glycerol, 6.5 mM potassium hydrogen phosphate and 6 mM magnesium sulphate was used for culturing the three strains of *Pseudomonas syringae.* The medium was supplemented with antibiotics corresponding to each strain (Rifampicin 100 µg/ml for *Pst* DC3000, Rifampicin 100 µg/ml and Tetracycline 10 µg/ml for *Pst* DC3000 *avrRpm1* and Rifampicin 100 µg/ml, Kanamycin 50 µg/ml and Spectinomycin 50 µg/ml for *Pst* DC3000 *COR*$^{-}$ AK87).

3.2.2. *Agrobacterium tumefaciens*

Agrobacterium tumefaciens C58C1 strain containing the pGV2260 plasmid carrying Rifampicin resistance gene was used throughout this work for transforming plants with constructs of interest (Deblaere *et al*., 1985) and was grown at 28°C in Luria Bertani (LB) medium containing 1% bacto triptone, 0.5% bacterial yeast extract and 1% sodium chloride supplemented with Rifampicin (100 µg /l).

3.2.3. *Escherichia coli*

E. coli DH5α strain (Hanahan, 1983) was used for transformation and propagation of plasmids of interest and was cultured at 37°C in LB medium.

3.3. Vectors for cloning

The following vectors were utilized throughout this study for introducing fragment DNA of interest into *E. coli*, *Agrobacterium* and plants

3.3.1. pGEM-T Easy vector

The pGEM-T Easy vector system (Promega) contains 3' Thymidine overhangs on which a poly Adenylated PCR product of interest is annealed during overnight ligation at 4°C. Upon transformation, the positive transformants were selected on plates with ampicillin and X-Gluc. White colonies formed were selected as the β-galactosidase genes present in the vector is interrupted on correct ligation of the insert.

3.3.2. pDONR201 vector

This vector (Invitrogen) was utilized hand in hand with the Gateway system of cloning, which is based upon the site specific recombination of bacteriophage λ in *E.coli*. An integration reaction is carried out in which a homologous recombination occurs between the PCR product of interest amplified with the *att*B (recombination site for bacteriophage λ) flanks, *att*b1 (5'- GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TNN-(insert sequence)-3') and *att*B2 (5'- GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTN-(insert sequence)- 3') and the pDONR201 plasmid carrying the *att*P sites. This results in the formation of an entry clone that can then undergo an excision reaction with a destination vector carrying *att*R sites. The by-product plasmid formed in both reactions carry *ccd*B gene which is lethal to *E.coli*. The positive clones are selected based on its resistance to the respective antibiotic marker present in the plasmid used.

3.3.3. pGWB1 vector

The pGWB vectors (Nakagawa *et al*., 2007) are destination binary vectors which are transformed to *A. tumefaciens* and then to plant. These vectors carry the *att*R sites necessary for

homologous recombination with the entry clone as described above. The resulting destination vector is selected based upon the kanamycin and hygromycin resistance present in it.

3.4. Preparation of competent cells

The preparation of *E. coli* DH5α cells was done by treatment with calcium chloride as described in Inoue *et al*. (1990).

In the case of competent C58C1 cells for *Agrobacterium tumefaciens*, a pre-culture of 5 ml was prepared in LB with Rifampicin from a colony in a fresh plate and was incubated overnight at 28°C. This pre-culture was diluted 40 times in a fresh LB medium with Rifampicin and incubated at 28°C for 5-6 hrs with agitation. The culture was then centrifuged at 5000 rpm for 10 min at 4°C and the resultant pellet was dissolved in 100 ml of sterile 100 mM Tris-HCl (pH 8). This suspension was again centrifuged 5000 rpm for 10 min at 4°C and the resultant pellet dissolved in 2 ml of LB, from which aliquots of 200 µl were frozen in liquid nitrogen and stored at -80°C until use.

3.5. Bacterial transformation

The transformation of competent *E. coli* DH5α cells was carried out by heat shock as described in Sambrook *et al*. (1989). The transformed cells were plated in LB with necessary antibiotics and incubated overnight at 37°C.

The transformation of competent *Agrobacterium tumefaciens* was carried out by adding 1 µg of plasmid DNA of interest into aliquots of 200 µl of competent cells. Then the aliquot was placed in ice for 5 min, followed by cold shock with liquid nitrogen for 5 min and then placed at 37°C for 5 min. 1 ml of LB was added and incubated at 28°C for 1 hr and 200 µl was plated in LB plates with antibiotics for selection and incubated at 28°C for 48 hrs.

3.6. Plant growth conditions

In the case of germination in soil, the seeds were sown in a mix of soil and vermiculite in the ratio of 3:1, stratified in dark at 4°C for 3 days, and then moved to growth chambers in which the temperature and humidity are maintained at 21°C and 60%, respectively, with 14 hr and 10 hr light and dark photoperiod and a light intensity of 6000 lux. Normally the plants were grown for 3-4 weeks prior to analysis.

For *in vitro* analysis, the seeds were sterilized in a solution containing 5% bleach and 0.01% Tween-20 for 7 minutes and then repeatedly washed in sterile milliQ water for 3 times and stratified in dark at 4°C for 3 days. The seeds were germinated in MS medium supplemented with 1% sucrose and the pH of the medium was adjusted to 5.7 using KOH. For vertical growth 1.5% agar was added and 0.7% agar was added for horizontal growth.

3.7. Bacterial assays in plants

3.7.1. Infiltration inoculation with *Pseudomonas syringae*

Prior inoculation, *Pseudomonas syringae* was streaked in a KB plate and grown for 24 hours and bacterial cells were harvested in 10 mM magnesium chloride and the optical density (O.D) was measured at 600 nm using a spectrophotometer. A culture of 1 O.D and 0.1 O.D corresponds to 10^7 and 10^6 colony forming units (cfu), respectively. A 1 ml syringe without needle was used for inoculating 3-4 week old leaves. The plants were watered well prior inoculation so that the soil in which the plants grow remains humid throughout the bacterial analysis. The inoculation of bacterial suspension was done by injecting bacteria directly into the apoplastic space through the abaxial side of the leaf. Three mature leaves were injected per plant. For *in planta* bacterial growth analysis an inoculum of 10^5cfu/ml was used, while a concentration of 10^6cfu/ml was used for symptom analysis and staining for ROS and cell death.

3.7.2. Spray inoculation with *Pseudomonas syringae*

Prior inoculation, fresh plates were streaked with *Pseudomonas syringae* and grown at 28°C for 24 hrs. The bacterial cells were harvested with 10 mM magnesium chloride and the concentration of the culture was adjusted to 10^8cfu/ml and 0.04% Silvet L-77 was added to the bacterial culture. The bacterial suspension was then transferred to a vaporizer and sprayed homogenously over the surface of mature 3-4 week old plants. The spray inoculated plants were then placed inside miniature greenhouses which consist of a tray with a lid to retain moisture. After 24 hrs post spray inoculation, the lids were removed and the symptoms and bacterial growth were quantified at 3 days post inoculation.

3.7.3. *In planta* bacterial growth analysis

The bacterial growth in leaves of *Arabidopsis* after infiltration inoculation was done as described in Whalen *et al*. (1991). In brief, 6 plants were inoculated and 18 inoculated leaves were pooled together and 3 leaves were randomly chosen to constitute an experimental sample. Leaf discs of 0.6 cm were cut from each inoculated leaf. Therefore, 6 experimental samples each consisting of 3 leaf discs were prepared per experiment in 500 µl of 10 mM magnesium chloride and homogenized in a robot (Retsch) with the help of 2 mm glass beads per each time point analyzed. The amount of bacterial growth was then estimated by plating different dilutions of the homogenate in KB plates with the corresponding antibiotic of selection. For every analysis, a minimum of three individual experiments were performed and the final results are an average of the same. In the case of spray inoculation, to minimize variability and for unbiased determination of bacterial growth, three leaves at the same stage of development in a rosette were chosen per plant across different genotypes. The rest of the bacterial growth determination was done as described in the section above.

3.8. *In vitro* analysis of plant responses

The response of plants to oxylipins, isoxaben and other compounds of interest were analyzed *in vitro* by germinating previously sterilized and stratified seeds in MS medium containing the compound of interest in growth chambers with a 14 hrs light period (5000 lux) at 21°C and 45% relative humidity. To study the root response phenotype to oxylipin treatment, the seeds were germinated vertically in MS plates containing 1.5% agar for 3 days and then, the seedlings at the same stage of growth were passed to a new MS plate with the respective oxylipin under study. Phenotype scoring was done after 3 days of growth in presence of oxylipins. The concentration of the oxylipin and the compounds used are specified in the legends of each figure. For RNA analysis, two methods were followed; in the first method the seeds were germinated in vertical plates for 12 days and then treated with the compound of interest in MS liquid and covered uniformly over the seedlings. Samples were then collected after specific time points. In the second method, seeds were germinated in horizontal plates for 10 days and then covered by the compound of interest as in the case of the first method and tissues collected at specific time points.

3.9. Stomatal assays

3.9.1. Measurement of stomatal apertures

Leaves at the same development stage were collected and placed in the stomatal buffer (50 mM KCl, 10 mM MES and 0.1 mM $CaCl_2$) for 2 hrs in Petri plates to make sure that the stomata were open before treatments (Melotto *et al*., 2006). After two hours, the buffer was replaced and the treatments were done and lower epidermal peals were prepared at specific time points. For every independent experiment 3 leaves were treated and three peals were then mounted in microscopic slides and then visualized in a microscope fitted with camera. For every peal, 20 representative pictures were taken with the Nomarski filter. The length and breadth of the stomatal aperture was measured using ImajeJ program and the stomatal aperture is indicated as a percentage ratio of the two values. For measuring stomatal apertures in response to bacteria, an inoculum concentration of 10^8cfu/ml was used, while the concentration of ABA and Oxylipins used were 10 μM and 10 nM respectively.

3.10. Histological analysis

3.10.1. Callose staining

Callose deposition after treatment with oxylipins was stained with Aniline Blue fluorochrome (Biosupplies, Australia) as described in Stone *et al*. (1984). Aniline blue has high specificity for complexing with β-1, 3-glucans of callose. For staining roots, a stock of 0.1 mg/ml was prepared in distilled water and then a working solution was prepared by diluting the stock in water at a ratio of 1:3. Roots were stained for 30 min in dark and then washed with water and visualized in microscope under UV light.

3.10.2. Cell death staining

The staining for cell death after bacterial inoculation was done by dye exclusion method as described in Hamberg *et al*. (2003). The staining solution consists of 10 ml lactic acid, 10 g phenol, 10 ml glycerol, 10 ml H_2O and 10 mg trypan blue. Trypan blue being a vital stain is able to traverse the cell membrane of dead cells and stain them blue without affecting the live cells. For staining dead cells, the trypan blue solution was diluted in the ratio of 1:1 with ethanol and the leaves were submerged into this solution and then heated in a microwave for 5 seconds, through which a

homogeneous staining of the leaves is obtained. The excess stain was removed with a solution of 5 g/2 ml of chloral hydrate in water

3.10.3. Hydrogen peroxide staining

Hydrogen peroxide production was visualized using 3, 3'- diaminobenzidine (DAB) (Sigma Aldrich) as described in Thordal-Christensen *et al*. (1997). DAB is a chromophore which generates a brown colored oxidation product in presence of H_2O_2. DAB staining was performed in mature leaves infected with bacteria by infiltration inoculation at 2 dpi. About 6-10 leaves from 3 individual plants were submerged in the DAB solution (1 mg/ml) per time point and stained in dark. After overnight uptake of the stain, the staining solution was removed and the leaves were dechlorophyllated by placing them in absolute ethanol for better visualization of the stain.

3.10.4. Lignin staining

Lignin, the complex constituent of secondary cell walls of plants was stained as described in Schrick *et al*. (2004) using a solution of Phloroglucinol in 20% HCl, which reacts with the cinnamaldehyde end groups of lignin to give a red color, for 10 min. The seedlings were then washed in water and visualized in microscope through bright field illumination.

3.10.5. Nitric oxide staining

In vivo production of nitric oxide was visualized using 4,5-diaminofluorescein diacetate (DAF-2DA) (Alexis biochemicals), a membrane permeable fluorescent probe which when inside the cell is hydrolyzed by esterases to 4,5-diaminofluorescein-2 (DAF-2) which in turn can react with nitric oxide to form the fluorescent DAF-2 triazole with excitation and emission wavelengths of 490 nm and 520 nm, respectively. For staining nitric oxide in the stomata, the epidermal peals were incubated in 15 μM staining solution prepared in the stomatal buffer solution for 30 minutes in dark and the peals were washed for 3 times before examination in microscope with the GFP filter (Melotto *et al*., 2006).

3.11. Extraction and analysis of nucleic acids.

3.11.1. Extraction of Plasmid DNA

For extracting plasmid DNA in the range of 20 µg from a 5-10 ml culture, a commercially available QIAPrep Spin Miniprep kit was used in which DNA binds to silica gel membrane in a spin column, whereas, a QIAGEN Plasmid Kit was used for culture volumes of 100 ml or more. The Plasmid DNA extracted was then quantified using a spectrophotometer in which 1 OD at λ_{260nm} corresponds to 50 mg/ml of DNA.

3.11.2. Extraction of Plant DNA

Plant DNA extraction was done by phenol-chloroform extraction method as described in Dellaporta *et al*. (1983). For higher number of samples, a Biosprint Plant DNA extraction kit from QIAGEN was used, in which high quality DNA was obtained using a technology which involves the use of Biosprint Robot and magnetic particles.

3.11.3. Amplification of DNA fragments

The amplification of a fragment DNA of interest was carried out through a Polymerase Chain Reaction (Saiki *et al*., 1985) in a thermocycler from GeneAmp PCR System 9700 of Perkin Elmer. The primers necessary for amplification of fragments of interest were bought from Sigma-Aldrich.

3.11.4. Restriction digestion of DNA

Fragment DNA of interest was also excised by using restriction endonucleases. Restriction digestion of DNA was done based on the specificity of enzyme used following the specifications of the supplying company.

3.11.5. Electrophoresis of DNA samples

The fragments of DNA obtained by PCR and restriction digestion were visualized by agarose gel electrophoresis using ethidium bromide in 0.5 X TBE buffer (45 mM Tris Borate, 1 mM EDTA, pH 8) which intercalates with the DNA and fluoresces under UV light.

3.11.6. Elution of DNA fragments from Agarose gels

The DNA fragments separated by Agarose gel electrophoresis were then eluted by using QIAquick Gel Extraction kit (Qiagen).

3.11.7. Sequence analysis of DNA

The sequencing of DNA was carried out at SECUGEN and the sequences were analyzed by DNASTAR Lasergene and Chromas softwares.

3.11.8. Extraction of RNA from Plants

The total RNA from plants was extracted using guanidium hydrochloride as described in Logermann *et al*. (1987). In brief, the plant tissue is homogenized in a solution of guanidium hydrochloride, EDTA and Morpholino Ethane Sulfonic acid (MES). Total RNA is extracted with a mix of phenol, chloroform and isoamyl alcohol (25:24:1). The RNA is precipitated from the extract, overnight at -20°C using 1 M acetic acid and 100% ethanol. The total RNA is then quantified using spectrophotometer in which 1 OD at λ_{260nm} corresponds to 40 mg/ml of RNA.

3.11.9. Semi-Quantitative RT-PCR

The alternative splicing leading to intron retention in *noxy76* mutant was confirmed through semi quantitative RT-PCR by treating RNA samples from wild type and *noxy76* with DNAse TURBO DNA-free (Ambion), for eliminating DNA contamination in the samples and Titan One Tube RT-PCR System (Roche Applied Science) for retro transcription of RNA. After retro transcription, the region of interest is amplified with specific primers and the amplicons were analyzed in an agarose gel.

3.11.10. Electrophoresis of RNA samples

Gel electrophoresis of RNA was done using denaturing formaldehyde agarose gels as described in Sambrook *et al*. (1989). In brief, 5 µg of RNA was denatured at 55°C for 15 mins in presence of formamide, formaldehyde and ethidium bromide and resolved in 1.5% agarose gels containing 15% formaldehyde and MEN buffer (Morpholino Propane Sulfonic acid, Sodium acetate and EDTA) and the electrophoresis was carried out in MEN buffer for 2 hrs at 100 v.

3.11.11. Blotting technique

RNA resolved in a gel is transferred into a Nylon membrane (Hybond-N, Amersham) through capillary transfer following the instructions provided by the supplying company. After capillary transfer, the nucleic acids were fixed on to the membrane by exposure to UV light (700 mJ/cm^2).

3.11.12. Radio labeling and hybridization of nucleic acids

The radiolabelled riboprobes were prepared by *in vitro* transcription in presence of α-[^{32}P] CTP, by using the RNA Transcription Kit (Roche). The template DNA was cloned in pGEMT-Easy vector which contains the promoter sequence necessary for annealing of the DNA polymerase. The vector with the template DNA was restriction digested anteriorly so as to prevent the transcription of the entire plasmid. In this manner, riboprobes were prepared for the *ABC* (encodes an ABC transporter, At1g15520), *POX* (FAD-dependent pyridine nucleotide-disulphide oxidoreductase, At5g22140), *FOX* (FAD-binding Berberine family protein with oxidoreductase activity, At1g26390), *TOUCH3* (Calmodulin-like protein At2g41100) and *MDC* (Mitochondrial dicarboxylate carrier protein, At2g22500).

The membranes containing the RNA are pre-hybridized with a solution containing 5 X SSC, 50% formamide, 0.25 g Milk powder, 0.5% SDS and 20 mg/ml of denatured salmon sperm DNA for a minimum of 2 hrs. After the addition of radiolabelled probes, the membranes were hybridized for a period of 8-15 hrs. Later on the membranes were washed for 25 min consecutively in solutions of decreasing salt concentrations (5 X SSC, 0.1% SDS and 2 X SSC, 0.1% SDS) and finally in a solution of 0.1 X SS, 0.1% SDS. All the hybridizations and washing steps were done at 68°C. The membranes were dried and autoradiographed on to photografic films of Amersham biosciences.

3.12. Genetic analysis

In order to analyze the genetic nature of the mutants under study, crosses were made between mutants and various ecotypes. Artificial pollination was done by opening the flowers in the bud stage and exposing the stigma without damaging it and removing all the other floral parts of one plant. The pollen grain from the other plant under study is dusted on the open stigma of the previous plant.

Also, in order to determine the character (dominant or recessive) of the mutation and to remove any additional mutations which might interfere with the one under study, the mutants were back crossed with wild type Col-0 several times and the F1 and F2 progeny were analyzed. Moreover, with the aim of localizing the mutation in the genome of *Arabidopsis*, a map-based cloning approach was adopted. The mutants which are in Col-0 background were crossed with ecotype L*er* which enabled the selection of F2 recombinant population. The high level of polymorphism between Col-0 and L*er* helped in performing the chromosome walking experiments which culminated in mapping the mutation. Molecular markers of the type SSLP (Simple Sequence Length Polymorphism; Bell and Ecker, 1994) and CAPS (Cleaved Amplified Polymorphic Sequences; Konieczny and Ausubel, 1993) which are present in the TAIR webpage for *Arabidopsis thaliana* database and Marker tracker from the University of Toronto were utilized for the same.

In the case of *noxy3*, the complete mapping process was performed in the laboratory. In the case of *noxy72* and *noxy76*, out-crosses with L*er* were done and the F2 recombinants were selected in the laboratory and sent to the mapping service at the División de Genética, Departamento de Biología Aplicada e Instituto de Bioingeniería, Universidad Miguel Hernández in Elche for first-pass mapping. Fine-resolution mapping and sequencing of candidate genes of *noxy76* was performed in the laboratory, while massive sequencing of the genome of *noxy72* was performed in BGI Genomics, Hong Kong. The locus At5g57300 identified through massive sequencing of *noxy72* was re-sequenced to confirm the identity of the mutation in *noxy72*.

3.13. Generation of transgenic plants

3.13.1. Complementation of *noxy3* mutation

The cDNA (RAFL-09-31-C19) of the gene *noxy3* (LON1 Protease- At526860) was obtained from RIKEN. The complete cDNA was amplified with the *att*B primers (*att*B1- 5'-CTTTATAAAACTCAATCGCAGAA-3' and *att*B2-5'-CTCAGTCTTCTTGTTTGTCATA-3') for cloning by Gateway System. The PCR product with the *att*B flanking sites was then cloned into pDONR201 and then to pGWB2, which contains the constitutive 35S promoter from the cauliflower mosaic virus. The binary vector pGWB1 was then transferred to *Agrobacterium tumefaciens* from which it can be transformed into mutant *noxy3* plants with the objective of complementing the mutation.

3.13.2. Transformation of *Arabidopsis thaliana*

The transformation of the constructs from *Agrobacterium tumefaciens* into *Arabidopsis* was carried as in Bechtold and Pelletier (1993), with the modifications specified by the same authors (Bechtold and Pelletier, 1998).

3.13.3. Selection of transgenic plants

The F1 transformants were selected by plating the seeds in a medium containing kanamycin (50µg/ml) and carbenicillin (100µg/ml). Carbenicillin was used to remove *Agrobacterium*. The transformants were then passed to soil and grown until the seed were collected. The F2 seeds were then plated in a medium containing kanamycin and the segregation of the transgene was observed for each transformants. The transformants which showed 3:1 segregation of the transgene which have a single copy of the transgene, were selected and passed to soil for collection of seeds. The resultant F3 seeds were checked for homozygosity and used for the studies.

3.14. Isolation of homozygous T-DNA lines

The T-DNA lines obtained from NASC were confirmed for their homozygosity through Polymerase Chain Reaction. Genomic DNA was isolated from a minimum of 30 plants and two PCR's were done. First, primers specific for the region of insertion in the gene were utilized to amplify the endogenous gene. Secondly, one primer from the gene and one primer specific for the T-DNA insertion were used for DNA amplification. If the plants are in homozygous condition, only one band is obtained during the second PCR, while no amplification occurs in the first endogenous PCR. If the plants are in heterozygous condition two bands, one in each of the PCR products is obtained.

Results

4. RESULTS

4.1. Characterization of the 9-HOT insensitive *noxy* mutants

Previous studies in our laboratory have indicated that the oxylipins from the 9-LOX pathway were able to induce root waving with lateral root growth arrest phenotype during an *in vitro* seedling assay (Vellosillo *et al*., 2007). Importantly, this phenotype was accompanied by activation of defense responses that were in part operated through the modifications of the cell wall, including the production of callose, a β-1, 3-glucan polymer controlling pathogen infection. In order to study the role of 9-LOX pathway, EMS mutants were isolated previously which were insensitive to the application of the 9-LOX derivative, 9-hydroxy octadecatrienoic acid (9-HOT). The characterization of these mutants can provide significant clues in deducing the responses mediated by the enzymes and products of the 9-LOX pathway. 9-HOT was unable to induce the root waving phenotype in the mutants as it did with wild type Col-0 and hence these mutants were named as *noxy* for *n*on-responding to *oxy*lipins. Three mutants, namely *noxy3*, *noxy72* and *noxy76* were chosen for further characterization of their responses to the application of 9-HOT, isoxaben and pathogen attack.

4.1.1. Phenotypic analysis in response to 9-HOT and isoxaben

Root waving phenotype induced by 9-HOT in wild type plants was not observed in any of the three *noxy* mutants under study (Figure 1 Panel B), thereby confirming their utility in the characterization of the 9-LOX signaling pathway. The formation of callose, a high molecular weight β-1, 3-glucan, is an important aspect of development and plant responses to stress conditions (Verma and Hong, 2001). 9-HOT was able to induce the production of callose in wild type plants, which was visualized through Aniline blue staining, as deposits in the region of the root which showed the waving phenotype, whereas the three *noxy* mutants were impaired in the production of callose in compliance with insensitivity of *noxy* mutants to 9-HOT (Figure 1 Panel C).

Besides, the response of the three *noxy* mutants were observed upon growth in isoxaben, a herbicide which inhibits the cellulose synthase thereby resulting in severe growth arrest characterized by root thickening and shortening (Desprez *et al*., 2002). Previous studies in the laboratory have shown that many *noxy* mutants isolated were partially insensitive to isoxaben. The three *noxy* mutants under observation were also partially insensitive to the application of Isoxaben

and did not exhibit the severe growth arrest and root swelling phenotype of wild type Col-0 seedlings. Additionally, lignification of roots was an important characteristic of wild type plants responses to isoxaben. The *noxy* mutants under characterization were also impaired in the formation of lignin (Figure 2).

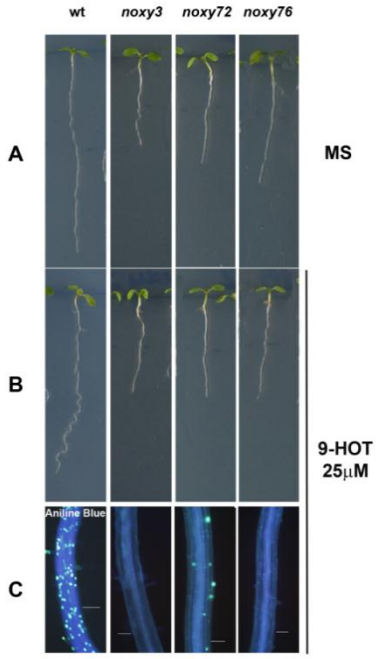

Figure 1. Phenotypic analysis of Col-0 and *noxy* mutants in response to 9-HOT

Phenotype of 7-day-old plants grown in MS for 3 days and then transferred to control MS medium (Panel A) and MS medium supplemented with 9-HOT (25 μM) (Panel B).

(C) Visualization of callose deposits in 9-HOT roots stained with aniline blue (All bars=50 μm).

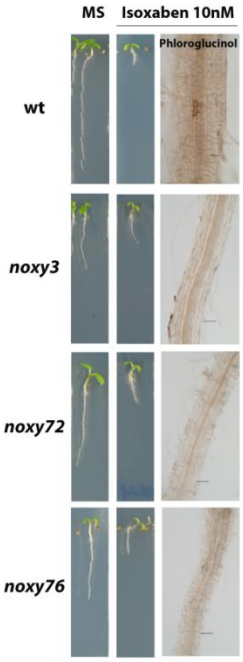

Figure 2. Phenotypic analysis of Col-0 and *noxy* mutants in response to isoxaben

Shown are 7-day-old plants grown in MS and MS supplemented with isoxaben (10 nM). Visualization of lignin deposition in isoxaben grown roots stained with phloroglucinol are shown in right panels (All bars=50 μm).

4.1.2. Characterization of *noxy* mutants based on their response to *Pseudomonas syringae*

The activation of the 9-LOX pathway during bacterial infection in leaves was highlighted in previous studies (Hamberg *et al*., 2003; Hwang and Hwang, 2010; López *et al*., 2011). Since we were interested in studying the role of 9-LOX pathway in defense responses, it was necessary to understand the effect of pathogens on these *noxy* mutants, as they might provide some insight into

the role of 9-LOX pathway in response to pathogen infection. Therefore, the responses of these *noxy* mutants to the phytopathogen, *Pseudomonas syringae* were analyzed.

Pseudomonas syringae often exhibits an epiphytic phase i.e., surviving on the plant surface before entering the plant apoplastic space, mainly through the stomata. So, it is necessary to study the defense responses mediated by the apoplast as well as the entry level defense response through stomatal closure. To begin with, the defense responses mediated in the apoplast were characterized in the three *noxy* mutants under study.

4.1.2.1. Characterization of apoplastic defense in *noxy* mutants

4.1.2.1.1. Apoplastic response of *noxy3* to *Pseudomonas syringae*

The response of *noxy3* mutant to *Pseudomonas syringae* was analyzed by infiltrating a suspension of *Pseudomonas syringae* directly into the apoplastic space. The quantification of the growth of the bacteria in response to inoculation with the two strains of bacteria, *Pst* DC3000 *avrRpm1* (avirulent) and *Pst* DC3000 (virulent) (Figure 3A) did not reveal any difference with respect to Col-0, suggesting that the mutant might be involved in aspect of 9-LOX pathway other than apoplastic defense responses.

Further examination was done to study the development of symptoms, cell death and H_2O_2 accumulation. The symptoms were compared with respect to wild type Col-0 at 3 days post inoculation. Inoculation with the *Pst* DC3000 *avrRpm1* leads to a hypersensitive response characterized by water soaked necrotic lesions in the region of inoculation, whereas in the case of inoculation with the virulent strain of bacteria, *Pst* DC3000, the symptoms observed were characterized by water soaked necrotic lesions surrounded by chlorotic tissue. The *noxy3* mutant did not show any significant difference in symptoms in comparison to Col-0 (Figure 3B).

Localized cell death visualized by tryphan blue staining did not reveal any significant differences between the two genotypes. Furthermore, ROS production visualized as H_2O_2 accumulation using DAB staining indicated no significant differences among *noxy3* and wild type plants (Figure 3B).

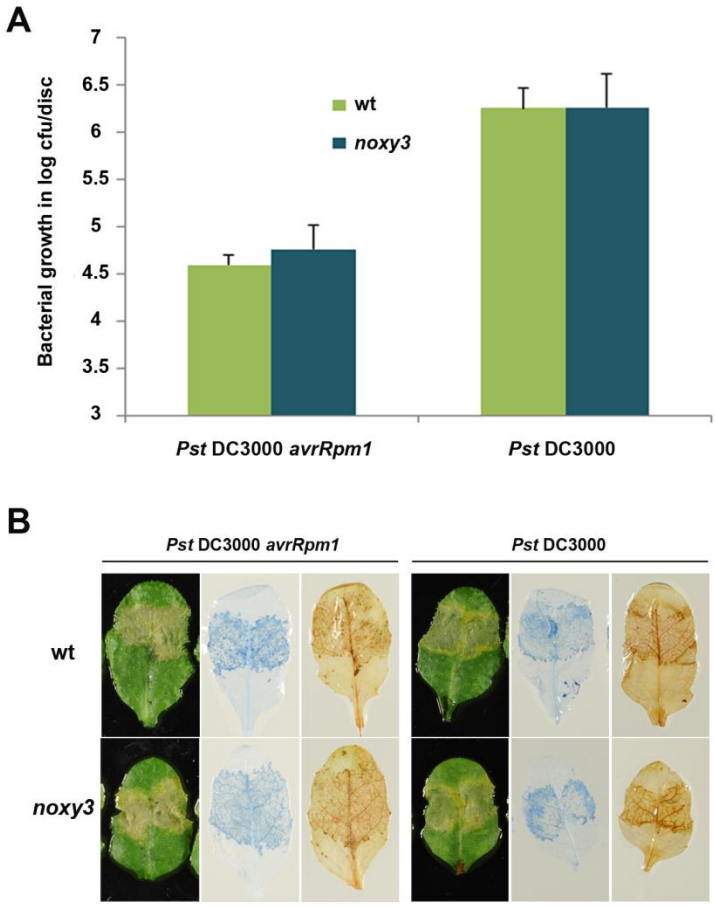

Figure 3. Analysis of apoplastic defense response of *noxy3*

(A) Growth of *Pst* DC3000 *avrRpm1* and *Pst* DC3000 in plants 3 days after bacterial infiltration (10^5 cfu/ml). Values are means and standard errors obtained in three independent experiments.

(B) Representative examples of lesions that developed in the leaves after bacterial infiltration (10^6 cfu/ml) (left column), cell death intensity stained with trypan blue (middle column) and H_2O_2 accumulation with 3,3'-diaminobenzidine (right column).

4.1.2.1.2. Apoplastic response of *noxy72* to *Pseudomonas syringae*

Analysis of the apoplastic response of *noxy72* to the two biotrophic strains of *Pseudomonas syringae* revealed that the bacterial growth was reduced in the *noxy72* mutant in comparison to wild type controls upon inoculation with the virulent strain of *Pseudomonas*. The *noxy72* plants harbored a 28% decreased growth of the virulent strain of bacteria in comparison to wild type plants (Figure 4A). Meanwhile no difference in bacterial growth was observed with respect to the avirulent strain.

The development of symptoms did not reveal any major differences with respect to avirulent strain of bacteria. But upon inoculation with the virulent strain of bacteria, the formation of water soaked necrotic lesions surrounded by chlorosis was prominent in wild type plants, whereas the *noxy72* mutant showed less chlorotic spots upon inoculation with the virulent strain (Figure 4B).

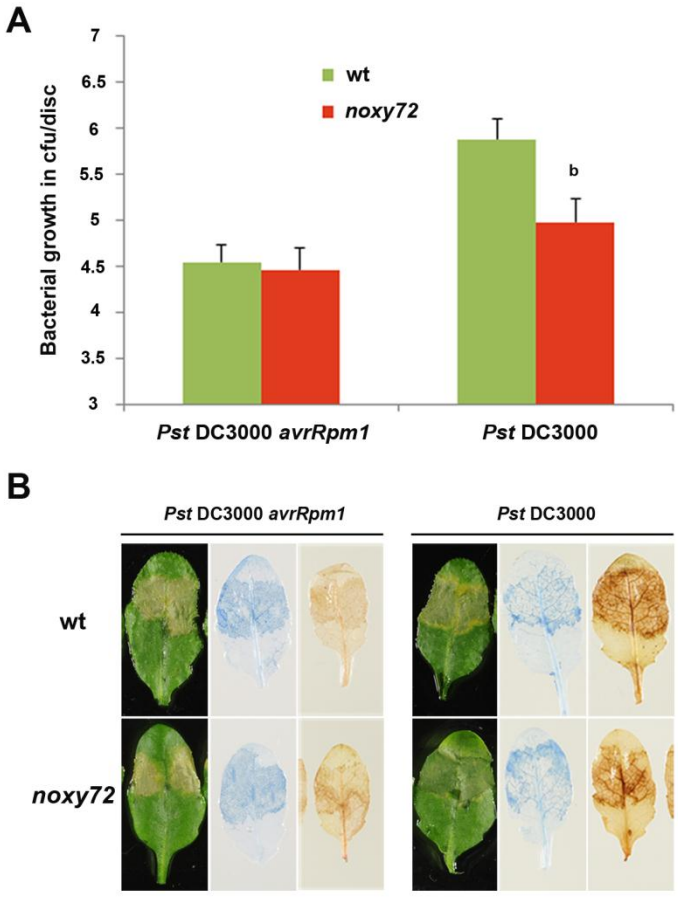

Figure 4. Analysis of apoplastic defense response of *noxy72*

(A) Growth of *Pst* DC3000 *avrRpm1* and *Pst* DC3000 in plants 3 days after bacterial infiltration (10^5 cfu/ml). Values are means and standard errors obtained in three independent experiments. Letters on top of the bars indicate statistically significant differences between *noxy72* and wild-type plants (Student's t test: $^b0.001 < P < 0.01$).

(B) Representative examples of lesions that developed in the leaves after bacterial infiltration (10^6 cfu/ml) (left column), cell death intensity stained with trypan blue (middle column) and H_2O_2 accumulation with 3,3'-diaminobenzidine (right column).

Furthermore, in the case of inoculation with the virulent strain of *Pseudomonas*, the analysis of cell death by trypan blue staining showed that the intensity of cell death was comparatively higher in *noxy72* than in wild type leaves (Figure 4B), suggesting a probable higher activation of cell death responsive genes in the *noxy72* mutant so as to limit the growth of the bacteria. In addition to this, the accumulation of hydrogen peroxide, which was visualized using DAB, was reduced in the *noxy72* mutant in comparison with the wild type plants (Figure 4B). However, no clear differences were observed in the case of the avirulent strain of *Pseudomonas*.

The fact that the *noxy72* mutant harbored less bacterial growth, hydrogen peroxide and increased cell death in the case of inoculation with the virulent strain of *Pseudomonas* indicates that the mutation leads to an activation of defense responses so that the mutant becomes more resistant to *Pst* DC3000 in comparison to wild type plants thereby highlighting the importance of this mutant in the study of defense responses and the role of 9-LOX pathway in activating defense responses against pathogen attack.

4.1.2.1.3. Apoplastic response of *noxy76* to *Pseudomonas syringae*

The quantification of bacterial growth revealed that *noxy76* permitted increased bacterial growth with 33% and 42% higher in comparison to wild type in the case of the avirulent and virulent strains of *Pseudomonas syringae*, respectively (Figure 5A). This higher degree of susceptibility also corroborated with the symptoms observed. Initial symptom analysis in response to both the strains of *Pseudomonas syringae* revealed that appearance of chlorotic lesions was accelerated in the *noxy76* mutant and strong symptoms were observed within 48 hrs of inoculation.

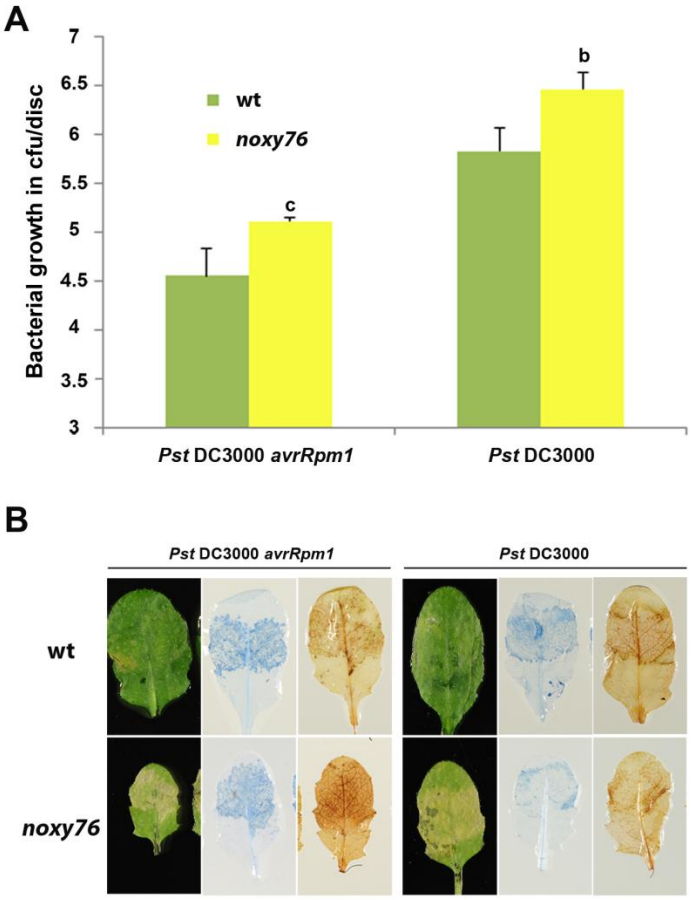

Figure 5. Analysis of apoplastic defense response of *noxy76*

(A) Growth of *Pst* DC3000 *avrRpm1* and *Pst* DC3000 in plants 3 days after bacterial infiltration (10^5 cfu/ml). Values are means and standard errors obtained in three independent experiments. Letters on top of the bars indicate statistically significant differences between *noxy76* and wild-type plants (Student's t test: $^b0.001 < P < 0.01$, $^c0.01 < P < 0.05$).

(B) Representative examples of lesions that developed in the leaves after bacterial infiltration (10^5 cfu/ml) (left column), cell death intensity stained with trypan blue (middle column) and H_2O_2 accumulation with 3,3'-diaminobenzidine (right column).

The symptoms were also characterized upon infection with a lower amount of inoculum (10^5 cfu/ml). In the case of wild type plants, practically no symptoms were observed except for the occurrence of few chlorotic spots. But in the *noxy76* mutant, strong chlorotic lesions in the region of inoculation were observed (Figure 5B).

For studying cell death and hydrogen peroxide formation, an inoculum concentration of 10^6 cfu/ml was used. Staining for cell death by trypan blue staining showed that the *noxy76* mutant had reduced cell death upon inoculation with the virulent strain of bacteria and staining for hydrogen peroxide revealed that *noxy76* accumulated more hydrogen peroxide in the case of inoculation with the avirulent strain of bacteria and (Figure 5B).

4.1.2.2. Stomatal defense responses in *noxy* mutants

The entry of pathogen into the plant is the first critical step during bacterial colonization of the host tissue. Foliar bacterial pathogens utilize the natural openings in the leaf surface mainly stomata, for gaining entry into the plant. Therefore, stomata form an important component of plant defense machinery. Previous studies have shown that the plants respond to surface inoculation with bacteria by closing the stomata. To counter act this, plant pathogens have evolved various strategies, including the ability to reopen the stomata. For the same, many pathovars of *Pst* DC3000 produce coronatine, a non-host specific phytotoxin which acts as a virulence factor and enhances the severity of disease symptoms by reopening the stomata and activating jasmonate responses (Melotto *et al*., 2006).

Recent data have thrown light on the ability of some 9-LOX oxylipins in inducing stomatal closure and thereby preventing the entry of pathogens (Montillet *et al*., unpublished). So it becomes interesting to study how the *noxy* mutants respond to surface inoculation of pathogen, which can provide some clues regarding the stomatal defense response mechanism in these mutants and highlight the role of 9-LOX pathway in stomatal defense responses.

Hence, the *noxy* mutants were surface inoculated by spraying with a suspension of either *Pst* DC3000 or *Pst* DC3000 *COR*$^-$ AK87. The latter is typically used for studying the stomatal defense responses as it does not produce coronatine and thus fails in reopening of stomata, as well as to examine the defense mechanisms of the plant that are triggered at the pre-invasion stage.

4.1.2.2.1 Stomatal defense response in *noxy3*

The plants were sprayed with *Pst* DC3000 and *Pst* DC3000 *COR*- AK87 strains of *Pseudomonas* at an inoculum concentration of 10^8 cfu/ml. At three days post inoculation, symptoms typical of *Pst* DC3000, chlorotic and necrotic lesions appeared (Figure 6A), while in the case of *Pst* DC3000 *COR*- AK87, minor symptom development occurred as partially chlorotic leaves were seen (Figure 6A). The symptom development did not vary significantly amongst the wild type and *noxy3* plants. But a 2.4 fold reduction was observed when the growth of the virulent strain was quantified and the reduction doubled to 5 fold in the case of the *Pst* DC3000 *COR*- AK87 strain in comparison to wild type Col-0 plants (Figure 6B).

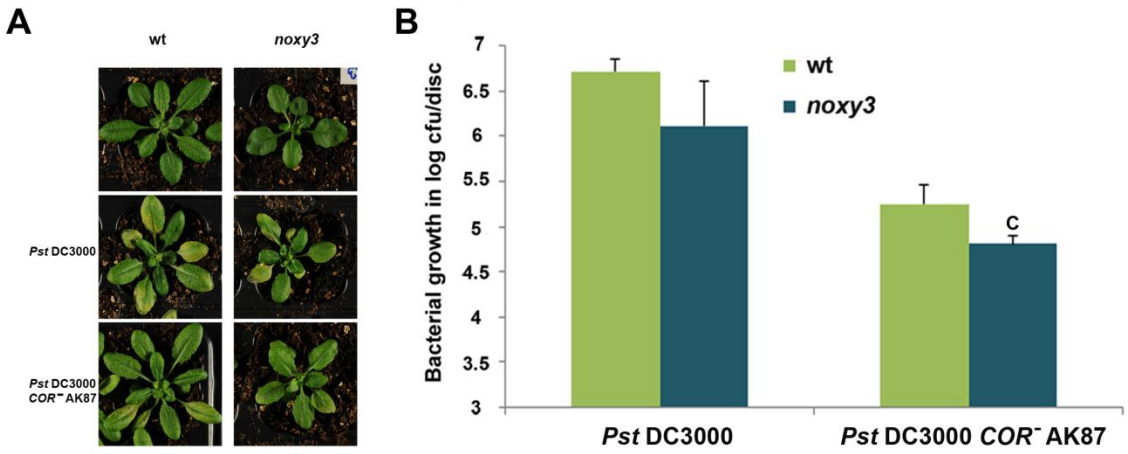

Figure 6. Analysis of stomatal defense response of *noxy3*

(A) Morphological phenotype of *noxy3* (top row) and disease symptoms (3 days post inoculation) of plants spray inoculated (10^8 cfu/ml) with *Pst* DC3000 (middle row) and *Pst* DC3000 *COR*- AK87 (bottom row).

(B) *Pst* DC3000 and *Pst* DC3000 *COR*- AK87 population at 3 days after spray inoculation at 10^8 cfu/ml. Values are means and standard errors obtained in 4 independent experiments. Letters on top of the bars indicate statistically significant differences between *noxy3* and wild-type plants (Student's t test: $^c 0.01 < P < 0.05$).

4.1.2.2.2. Stomatal defense response in *noxy72*

The analysis of the symptoms in response to spray inoculation with *Pst* DC3000 and *Pst* DC3000 *COR*- AK87 did not show any differences among *noxy72* and wild type Col-0 (Figure 7A) and the quantification of bacterial growth also did not reveal any significant difference between *noxy72* and wild type Col-0 plants in the case of *Pst* DC3000 *COR*- AK87, while a non-significant reduction of 1.7 fold was observed in the case of *Pst* DC3000 strain (Figure 7B).

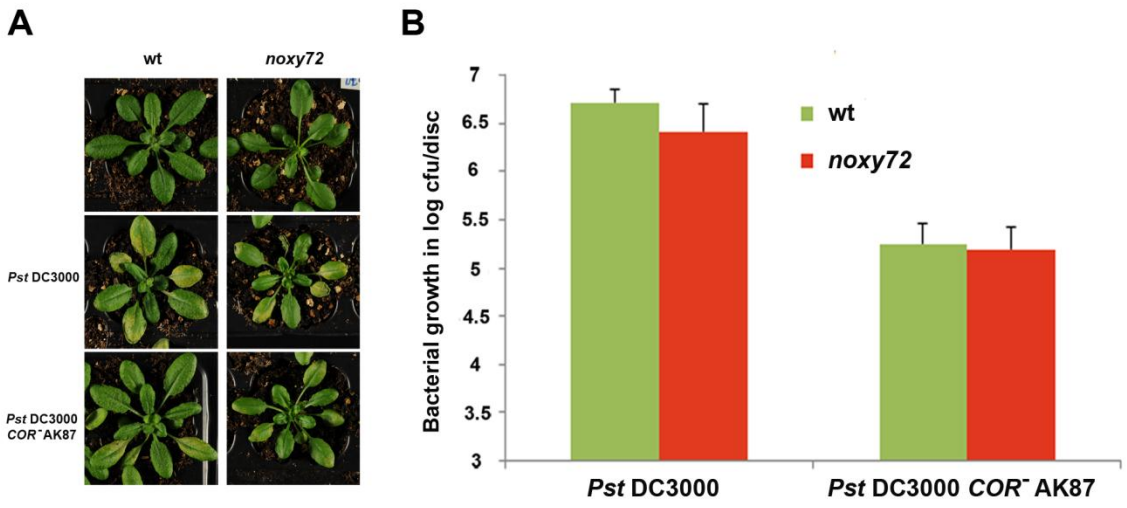

Figure 7. Analysis of stomatal defense response of *noxy72*

(A) Morphological phenotype of *noxy72* (top row) and disease symptoms (3 days post inoculation) of plants spray inoculated (10^8 cfu/ml) with *Pst* DC3000 (middle row) and *Pst* DC3000 *COR*⁻ AK87 (bottom row).

(B) *Pst* DC3000 and *Pst* DC3000 *COR*⁻ AK87 population at 3 days after spray inoculation at 10^8 cfu/ml. Values are means and standard errors obtained in 4 independent experiments.

4.1.2.2.3. Stomatal defense response in *noxy76*

The mutant *noxy76* which exhibited a defective apoplastic defense response thereby permitting increased bacterial growth was also defective in stomatal defense responses. Upon spray inoculation with both the strains of bacteria, the severity of the disease symptoms was stronger in this mutant in comparison to wild type plants. When inoculated with *Pst* DC3000, the *noxy76* mutant was hyper susceptible and plants show severe water soaked necrotic and chlorotic lesions and growth arrest (Figure 8A). To get a clearer picture on the development of disease, the bacterial growth was quantified on the 2nd day post inoculation, at which time the mutant plants still remain viable. The quantification of the growth of the virulent strain showed that the bacterial growth was 27 fold higher in the *noxy76* mutant (Figure 8B).

Besides this, the severity of the symptoms upon spray inoculation with *Pst* DC3000 *COR*⁻ AK87 were milder with chlorotic lesions and hence permitting the quantification at 3 days post inoculation. The bacterial growth was 11 fold higher in the *noxy76* mutant (Figure 8B) suggesting that this mutant is severely compromised in its responses to bacteria in the surface and hence this mutant can provide critical clues to the role of 9-LOX pathway in stomatal defense responses.

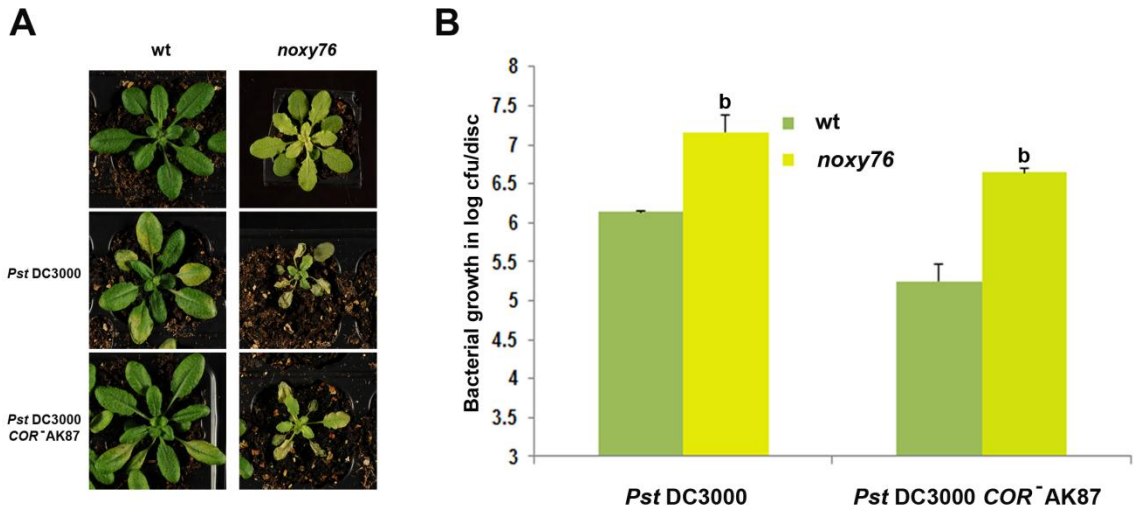

Figure 8. Analysis of stomatal defense response of *noxy76*

(A) Morphological phenotype of *noxy76* (top row) and disease symptoms of plants spray inoculated at 10^8 cfu/ml with *Pst* DC3000 (2 days post inoculation) (middle row) and *Pst* DC3000 *COR*⁻ AK87 (3 days post inoculation) (bottom row).

(B) *Pst* DC3000 and *Pst* DC3000 *COR*⁻ AK87 population at 2 and 3 days, respectively, after spray inoculation at 10^8 cfu/ml. Values are means and standard errors obtained in 3 independent experiments. Letters on top of the bars indicate statistically significant differences between *noxy76* and wild-type plants (Student's t test: $^b0.001 < P < 0.01$).

In summary, the results of these analysis indicated that *noxy3* was not altered in the apoplastic defense responses as it did not show any significant differences in comparison to wild type Col-0, while it showed increased resistance to surface inoculation with the coronatine deficient strain indicating the higher activation of stomatal defenses. In the case of *noxy72*, the apoplastic defense response was stronger and hence showed increased resistance to the virulent strain of *Pseudomonas syringae* upon infiltration inoculation, but no differences were observed during surface inoculation with the virulent and coronatine deficient strains of *Pseudomonas.* The mutant *noxy76* showed significant differences in comparison to wild type plants in both defense responses (Stomatal and Apoplastic). The bacterial growth analysis revealed that this mutant was highly susceptible to the three strains of bacteria used and hence compromised in activating apoplastic and stomatal defense responses (Table 1).

The characterization of the three *noxy* mutants based on their responses to *Pseudomonas* has enabled us to position these mutants in their corresponding place in the two layers of defense identified till date (Pre-Invasive and Post-Invasive stages) (Figure 9). *NOXY3* could be a negative

regulator of pre-invasive defense responses since the mutation in this gene activated defense responses leading to resistance. Since *noxy72* exhibited an enhanced apoplastic defense response, *NOXY72* could be a negative regulator of post-invasive defense responses. As *noxy76* exhibited susceptibility to both type of defense responses, *NOXY76* could invariably be a positive regulator of defense responses in *Arabidopsis.* The mapping of these mutants can open up new vistas in signaling responses to *Pseudomonas* mediated by the 9-LOX oxylipin pathway.

TABLE 1. Summary of responses of *noxy* mutants to bacterial inoculation

Response			*noxy3*	*noxy72*	*noxy76*
Apoplastic defense	*Pst* DC3000 *avrRpm1*	Bacterial growth	Similar to wt	Similar to wt	**Susceptible**
		Symptoms	Similar to wt	Similar to wt	**Strongly chlorotic**
		H_2O_2 accumulation	Similar to wt	Similar to wt	**Comparatively higher**
		Cell death	Similar to wt	Similar to wt	Similar to wt
	Pst DC3000	Bacterial growth	Similar to wt	**Resistant**	**Susceptible**
		Symptoms	Similar to wt	**Comparatively less chlorosis**	**Strongly chlorotic**
		H_2O_2 accumulation	Similar to wt	**Comparatively less**	Similar to wt
		Cell death	Similar to wt	**Comparatively higher**	**Comparatively less**
Stomatal defense	*Pst* DC3000 *COR*- AK87	Symptoms	Similar to wt	Similar to wt	**Strongly chlorotic**
		Bacterial growth	**Resistant**	Similar to wt	**Susceptible**
	Pst DC3000	Symptoms	Similar to wt	Similar to wt	**Strongly chlorotic**
		Bacterial growth	**Partially resistant**	Similar to wt	**Susceptible**

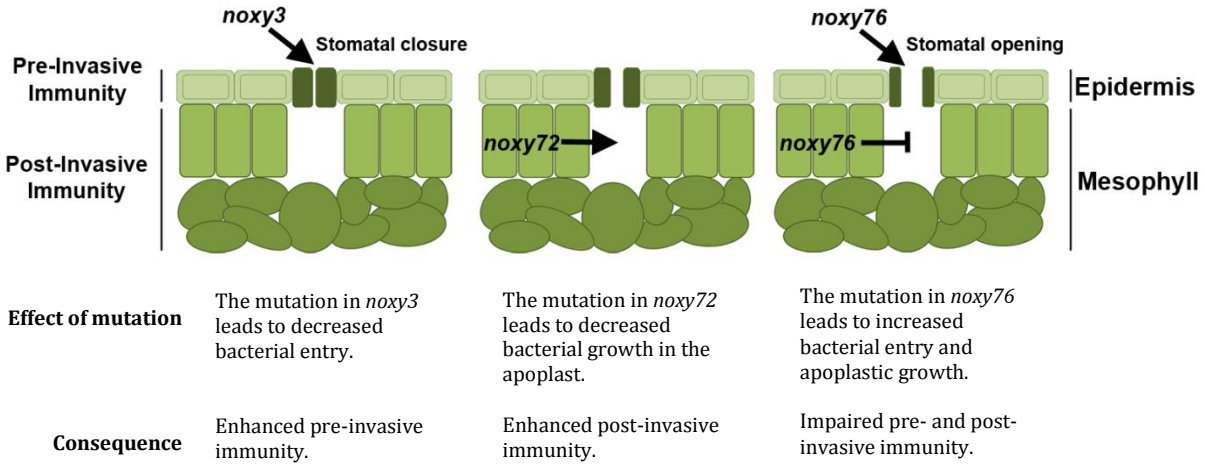

Figure 9. Schematic representation of the layers of defense responses in the *noxy3*, *noxy72* and *noxy76* mutants

Arrowhead indicates stimulation, while hammerhead indicates inhibition. *noxy3* restricts the entry of bacteria and hence has enhanced pre-invasive defense. *noxy72* behaves like wild type in stomatal response but potentiates a stronger defense response in the intercellular spaces. *noxy76* fails in activating pre-invasive defense as well as defense responses in the intercellular spaces, thereby exhibiting impaired responses at both layers of defense.

4.2. Map based cloning of *noxy* mutants

4.2.1. Map based cloning of *noxy3*

For localizing the mutation in the *noxy3* mutant, a map based cloning approach was employed, wherein the mutant *noxy3* in the Col-0 background was outcrossed with another ecotype, namely L*er*. Simultaneously, the mutant was also back crossed 3 times with wild type Col-0, so as to clean any other mutations which might impede its characterization. The partial insensitivity of this mutant to isoxaben was an important facet in identifying the recombinant populations in the chromosome walking experiments to identify the mutation. The F_1 population was then plated in isoxaben and the resultant phenotype corresponded to the wild type phenotype displaying sensitivity to isoxaben, and thereby confirming the recessive trait of the mutation. The screening of F_2 recombinants was done based upon the partial insensitivity phenotype to isoxaben which revealed that the mutation was recessive in character as the segregation of wild type and mutant corresponded to 3:1. The DNA from 560 F_2 recombinant individuals was analyzed through

Polymerase Chain Reaction, utilizing SSLP, dCAPS and MSAT markers, which revealed the polymorphism between the mutant and wild type backgrounds. Through this method, the mutation was narrowed down to be in a region of 33 genes in the F2P16 BAC present in the Chromosome 1. Sequencing of candidate genes was undertaken and the mutation in *noxy3* was localized to the 12th exon of At5g26860, in which 2 nucleotidic changes of G to A were observed at positions 1322 bp and 1330 bp (Figure 10). These mutations led to corresponding changes in the 441 amino acid (Arginine to Lysine) and the 444 aa (Glutamate to Lysine). The *noxy3* protein is encoded by a 5953 bp gene with 19 exons. The ultimate transcript is 2823 bp long encoding a predicted protein of 940 aa.

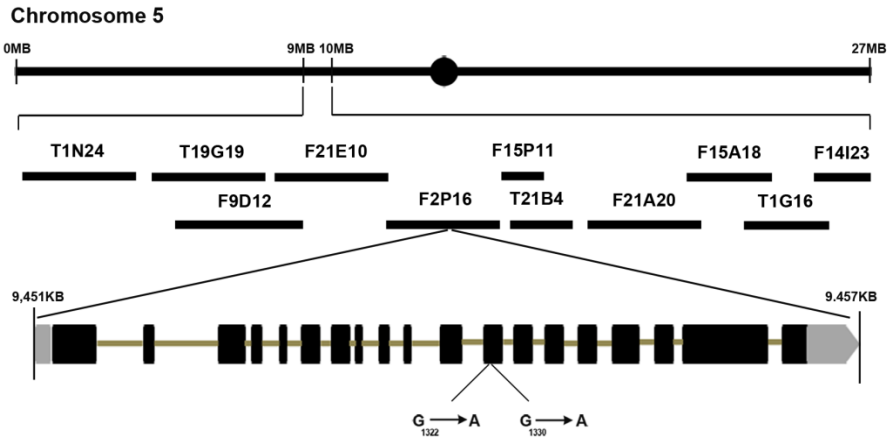

Figure 10. Map-based cloning of *noxy3*

Schematic representation of the mapping strategy followed to identify the *noxy3* mutation and structure of the *NOXY3* gene. Sequencing of candidate open reading frame from the genomic sequences of wild type and *noxy3* revealed two point mutations (G-to-A transition) at base positions 1322 and 1330.

4.2.1.1. Characterization of the locus At5g26860

The locus At5g26860 encodes the Lon1 protease which belongs to a family of AAA$^+$ proteases (ATPases associated with diverse cellular activities). The analysis for conserved domains on the NCBI Conserved Domain search portal (Marchler-Baeur *et al*., 2011) in this protein sequence revealed that the amino acid sequence in the region between 439 and 602 contained the motif corresponding to the AAA$^+$ type superfamily of enzymes (Figure 11A). The members of this family of enzymes share a highly conserved ATPase domain which conveys the free energy released by ATP hydrolysis to activities related to protein turnover in the organelles. Furthermore, the region between 729 and 903 aa contains the Chl 1 domain which is also known to contain ATP binding

sites, while the region between 101 and 903 aa contain the LON domain, which is found in the ATP dependent bacterial La protease.

While the ubiquitin/26S proteasome is necessary for protein turnover in the protoplasm and nucleus, plant organelles possess a distinct quality control system consisting of a range of ATP-dependent proteases belonging to the Clp, FtsH and Lon families (Rigas *et al*., 2009a). Lon proteases are multifunctional ATP dependent enzymes which are present in bacteria and organelles of eukaryotes. Lon was named after the *lon*g filament phenotype of *E. coli* K-12 mutant cells. The *LON* gene in *E. coli* encodes an AAA+ protease.

Arabidopsis has 4 distinct Lon proteases like proteins, namely Lon1, Lon2, Lon3 and Lon4, which are predicted to be localized in different cellular organelles. The Lon1 protein is targeted to the mitochondria as predicted in MitoProtII web portal, which gives a 95% probability of this protein to be targeted to the mitochondria. The localization of this protein to the mitochondria was experimentally proved by Rigas *et al*. (2009). Lon2 is predicted to be localized in the peroxisome and Lon3 in the mitochondria. Lon4 is known to localize in the chloroplast as well as in the mitochondria.

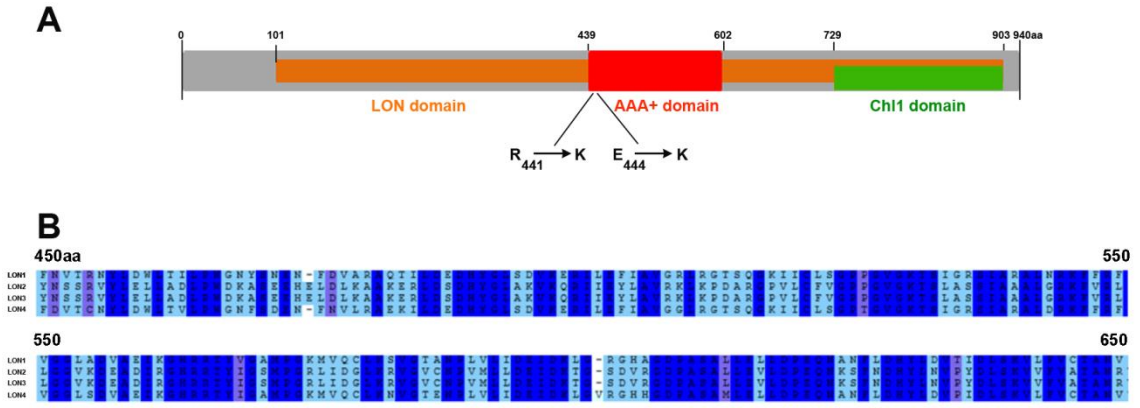

Figure 11. Representation of the domains identified in NOXY3

(A) Domain topology of NOXY3 predicted using NCBI Conserved Domain search portal. Two amino acid conversions (Arg-441 to Lys-441 and Glu-444 to Lys-444) were identified at the ATP binding domain of NOXY3.

(B) Alignment of AAA+ domains from the 4 Lon proteases identified in Arabidopsis.

A multiple sequence alignment of the 4 Lon proteases in *Arabidopsis* shows that there is a significant amount of amino acid conservation in the AAA+ module (Figure 11B), suggesting that these proteins are closely related to each other.

In yeast and mammals, Lon proteases are necessary for the control of selective turnover of non-assembled or misfolded proteins in mitochondrial matrix. In plants, two roles have been associated with Lon proteases. In *Zea mays*, Lon protease degrades the mitochondrial peptide, ORF239, which is associated with cytoplasmic male sterility (Sarria *et al*., 1998) while in *Arabidopsis*, Lon1 is specifically needed for mitochondrial function during post germinative growth leading to seedling establishment (Rigas *et al*., 2009).

4.2.1.2. Complementation of *noxy3*

To confirm whether the identified locus At5g26860 carries the mutation in *noxy3*, a wild type copy of the gene was expressed in the mutant background. For that, a transgenic construct, *NOXY3:NOXY3* carrying the wild type copy of the gene under its own promoter was built. The T3 homozygous transgenic lines were then tested for their response to 9-HOT (Figure 12) and isoxaben (Figure 13). Transgenic *NOXY3:NOXY3* plant exhibited the root waving and root growth arrest phenotype similar to wild type plants. Further histological analysis for callose deposition showed that the complementation was achieved as the aniline blue staining was identical to wild type Col-0.

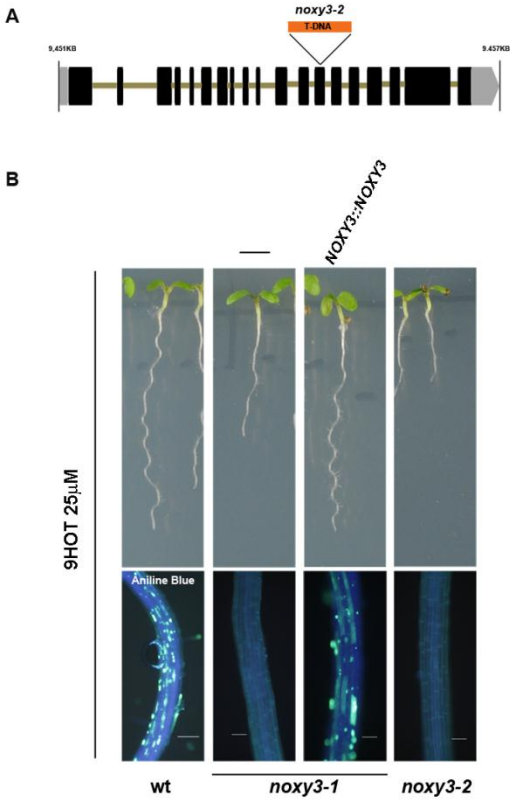

Figure 12. Complementation analysis of *noxy3* phenotype

(A) Schematic representation of the genomic sequence of *NOXY3* with exons represented as black bars. The position of the T-DNA insertion is shown.

(B) Phenotype of wild type Col-0, *noxy3-1*, *noxy3-1* transformed with *NOXY3::NOXY3* and the T-DNA insertion mutant *noxy3-2* grown in MS for 3 days and then transferred to control MS medium supplemented with 9-HOT (25 µM) (top row). Fluorescence images of callose deposits induced in 9-HOT roots stained with aniline blue (bottom row).(All bars=50 µm).

Besides the generation of transgenic lines for complementing the mutation, further confirmation of identified locus as the mutated locus was done through analysis of T-DNA insertion mutants of the identified locus. T-DNA insertion mutant (SALK_013817) designated as *noxy3-2* was obtained from NASC. This mutant carries a T-DNA insertion in the 13th exon of the gene (Figure 12A). Phenotypically this T-DNA mutant had roots shorter than *noxy3* and was designated as *noxy3-2*. Characterization based on responses to 9-HOT and isoxaben was carried out and *noxy3-2* also exhibited the *noxy3* mutant phenotype i.e., insensitivity to 9-HOT and partial insensitivity to isoxaben, thereby confirming the identified locus as the mutated locus (Figure 12B and Figure 13).

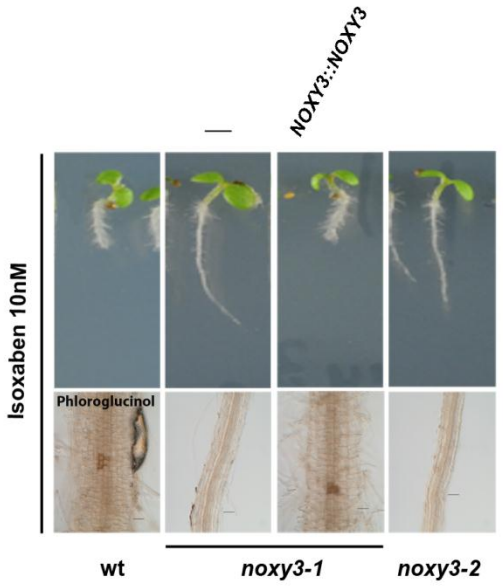

Figure 13. Complementation analysis of *noxy3* phenotype in response to Isoxaben

7-day-old seedlings germinated and grown in MS medium with 10 nM isoxaben (upper panel) and transmitted light images of Lignin deposition in isoxaben roots stained with Phloroglucinol (All bars=50 µm)(lower panel).

Under normal growing conditions *noxy3-1* mutant had shorter root (Figure 1A) and is smaller than wild type Col-0 plants (Figure 6A). The T-DNA insertion, *noxy3-2* showed a stronger root shortening phenotype (Figure 12).

4.2.2. Map based cloning of *noxy72*

Map based cloning strategy was also employed to map the mutation in another 9-HOT insensitive mutant, namely *noxy72*. *noxy72* was also outcrossed with L*er* ecotype and the resultant F_1 population was plated in isoxaben. All seedlings exhibited a wild type phenotype confirming the recessive nature of the mutation. Genomic DNA was isolated from 500 F_2 recombinant individuals which carried the *noxy72* root phenotype in presence of isoxaben. Molecular markers polymorphic

between Col-0 and L*er* defined the position of the mutation in a 140 Kb region in the chromosome 5. Upon massive sequencing, the mutation was identified to be the locus At5g57300.

The mutation corresponds to a change in a guanine nucleotide at position 94 in the 1st exon to an adenine nucleotide. This nucleotidic change in turn converts the 32 aa from Glycine to Arginine (Figure 14A).

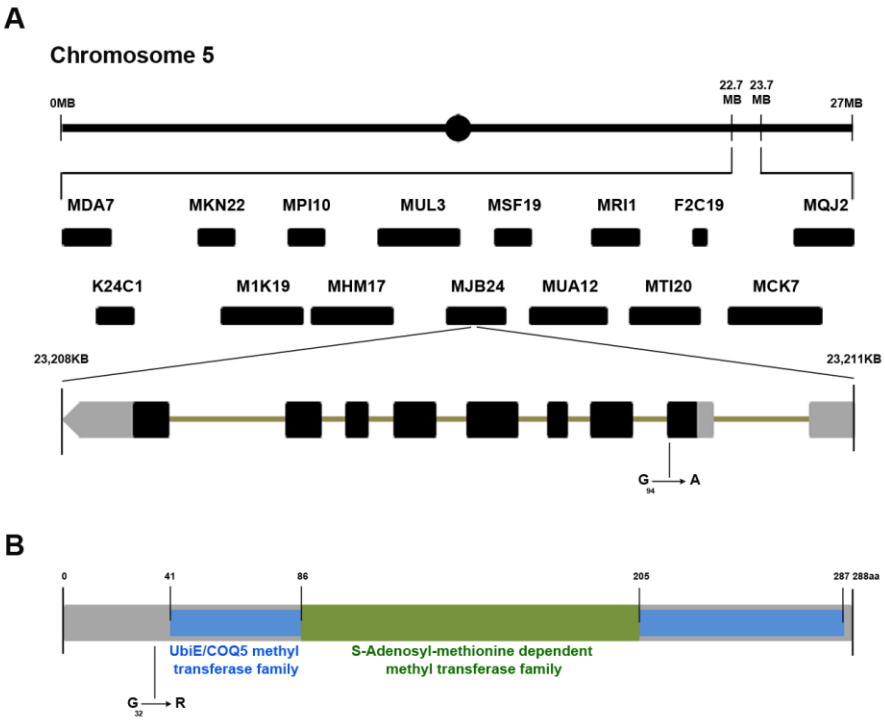

Figure 14. Map-based cloning of *noxy72*

(A) Schematic representation of the mapping strategy followed to identify the *noxy72* mutation and structure of the *NOXY72* gene. Sequencing of candidate open reading frame from the genomic sequences of wild type and *noxy72* revealed a single point mutation (G-to-A transition) at base position 94.

(B) Domain topology of NOXY72 predicted using NCBI Conserved Domain search portal. Amino acid conversion from Gly-32 to Arg-32 occurs in *noxy72*.

4.2.2.1. Characterization of locus At5g57300

The locus At5g57300 encodes a 2.5 Kb gene with 8 exons and the transcript is 867 bp long which gets translated into a protein of 288 aa. A search for conserved domains in the amino acid sequence reveals the presence of a sequence which is highly conserved in the S-adenosylmethionine-dependent methyltransferases family of protein in the region between the amino acids 86 and 205 (Figure 14B). These enzymes use S-adenosylmethionine for transferring

methyl group to form S-adenosyl-L-homocysteine. Furthermore the region between the amino acids 41 and 287 shared considerable sequence similarity with the UbiE/COQ5 methyltransferase family protein. The UbiE/COQ5 is necessary for the formation of 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinone from 2-polyprenyl-6-methoxy-1,4-benzoquinone, which is the penultimate step in the formation of Ubiquinone (KEGG Pathway database; Dibrove *et al*., 1997). Ubiquinone is an important component of the oxidative phosphorylation machinery, acting as an electron carrier. The analysis of the protein sequence using MitoProt II predicts that the protein has a 99% probability for being imported into the mitochondria.

Phenotypically, *noxy72* did not exhibit any alterations in comparison to wild type Col-0 and complementation studies and characterization of SALK T-DNA lines are in progress.

4.2.3. Map based cloning of *noxy76*

Similar to *noxy3* and *noxy72*, a map based cloning approach was adapted to map the mutation by crossing the *noxy76* mutant with *Arabidopsis thaliana* polymorphic ecotype L*er*. The analysis of the F_1 population revealed that the mutation is recessive in nature. About 670 F_2 individuals were identified based on the partial insensitivity phenotype to isoxaben and genomic DNA was isolated from them. Molecular markers polymorphic between Col-0 and L*er* ecotypes defined the position of *noxy76* in chromosome 1. Fine mapping using additional markers from dCAPS and MASC positioned the mutation in a region of 60 genes. Candidate genes were selected for sequencing and the mutation was localized in the 22nd intron of At1g64790 (Figure 15A).

4.2.3.1. Intron retention leads to altered mRNA in *noxy76*

Intron retention, a form of alternative splicing (Reddy *et al*., 2007) occurs in *noxy76*. The mutation identified in *noxy76* corresponds to a change from guanine to adenine at the end of the 22nd intron, which is a recognition site of the plant spliceosome machinery. Hence we predicted that insensitivity of this mutant to 9-HOT arose due to altered protein caused by intron retention. To prove that the intron retention does occur in *noxy76*, a fragment of the mRNA encompassing the mutated intronic region corresponding to 464 bp was reverse transcribed and amplified in wild type Col-0 and *noxy76*. An amplicon of 464 bp was obtained in the case of wild type as expected whereas an amplicon of 542 bp was obtained in the case of *noxy76* (Figure 16A). The 78 bp difference between the two amplicons corresponded to the size of the 22nd intron which is retained in the *noxy76* mutant due to the mutation at the spliceosome recognition site. The amplicons were

then sequenced and *noxy76* carried this 78 bp intronic region whereas it was absent in the case of wild type Col-0, thereby confirming the 22nd intron is retained in *noxy76* (Figure 16B).

The *noxy76* is encoded by an 18 Kb gene with 59 introns that are spliced out to yield an 8 Kb transcript and a predicted protein of 2696 amino acids (Figure 15B). Due to intron retention, a protein of 2722 aa is predicted to be translated from the *noxy76* mRNA.

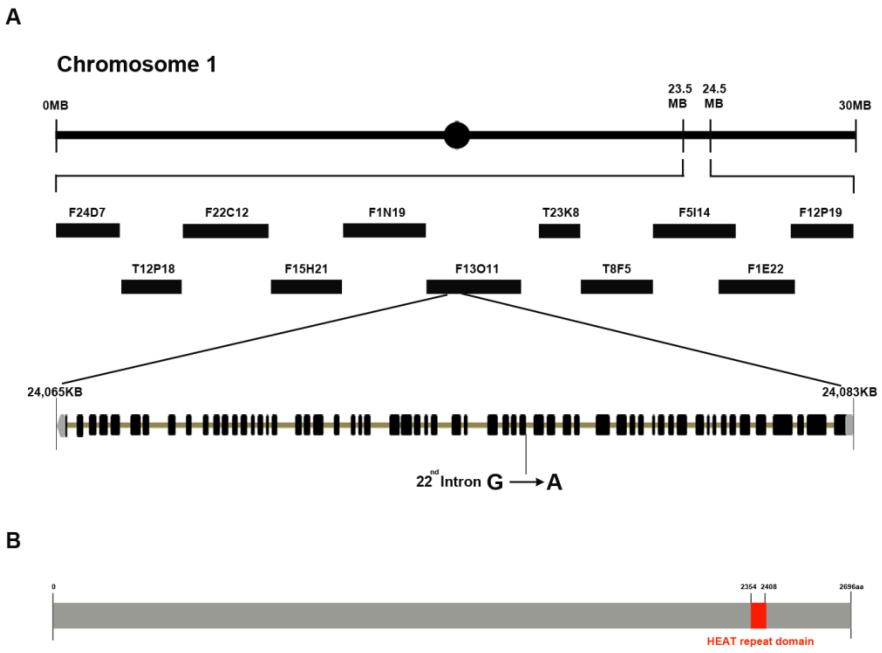

Figure 15. Map-based cloning of *noxy76*

(A) Schematic representation of the mapping strategy followed to identify the *noxy76* mutation and structure of the *noxy76* gene. Sequencing of candidate open reading frame from the genomic sequences of wild type and *noxy76* revealed a point mutation (G-to-A transition) at the last base of 22nd Intron.

(B) Domain topology of NOXY76 predicted using NCBI Conserved Domain search portal.

4.2.3.2. Characterization of locus At1g64790

The locus At1g64790 encodes a protein called as ILITYHIA, named after the Greek goddess of child birth. This gene is a translational activator and was first identified through a transcriptomic analysis of genes involved in female gametophyte and embryo sac development (Johnston *et al.,* 2007). Furthermore, ILITYHIA shares significant homology with yeast GCN1 (General Control of Nonderepressible1) protein which is necessary to release the translational repression of GCN4.

GCN4 is a transcription factor inducing the expression of amino acid biosynthesis encoding genes during amino acid starvation conditions in yeast (Marton *et al*., 1993).

In a previous study to characterize *ILITYHIA*, T-DNA insertion mutants showed a clear and distinct phenotype with the mutant plants being smaller than wild type Col-0 displaying serrated leaves that are yellow to light green in color (Monaghan *et al*., 2010). A milder but similar phenotype was observed in *noxy76* plants.

The search for conserved domains in this protein showed a single HEAT (Huntington's, Elongation factor EF3, protein phosphatase2A and yeast PI3-kinase TOR1) repeat domain in the region between 2354 and 2408 aa (Figure 15B).

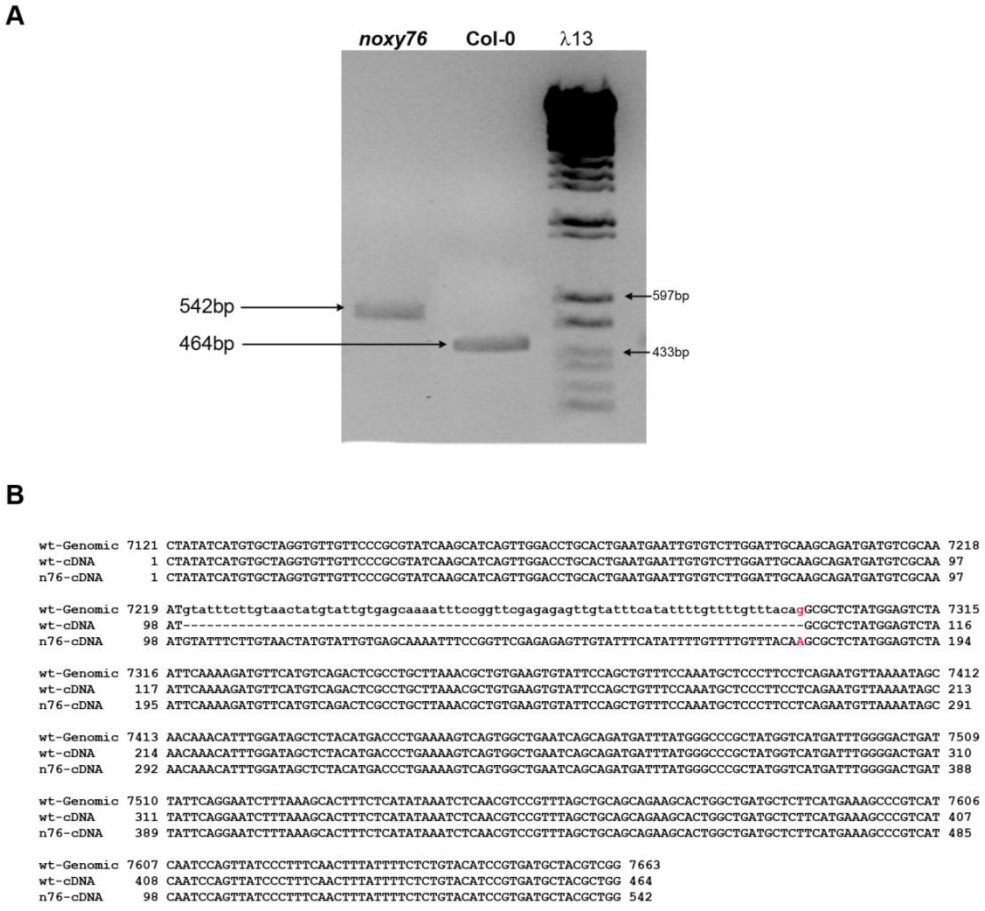

Figure 16. Intron retention in *noxy76*

(A) Semi-quantitative RT-PCR analysis of the fragment of the mRNA encompassing the mutated intronic region showing the distinct transcripts produced in wild type Col-0 and *noxy76* seedlings.

(B) Sequence of the cDNA fragments generated from wild type Col-0 and *noxy76* RNA samples.

4.3. Characterization of *noxy76*

The mapping and characterization of *noxy3, noxy72 and noxy76* has enabled us to partially annotate the probable role of these proteins in plant defense responses mediated by the 9-LOX pathway. *noxy3* was mapped to a mitochondrial Lon protease which has an enhanced pre-invasive immunity, against *Pseudomonas* defective in the production of coronatine. *noxy72* was mapped to mitochondrial methyl transferase enzyme involved in the production of ubiquinone and exhibited enhanced apoplastic immunity. On the other hand, *noxy76* exhibited susceptibility at both layers of immune response with the degree of susceptibility higher in the case of pre-invasive immunity. It was later mapped to *ILITYHIA*.

Out of the 3 *noxy* mutants initially characterized based on their defense responses, a detailed characterization of *noxy76* was undertaken as it exhibited strong defects in apoplastic and stomatal defense responses and will enable us to identify the role played by this gene during defense signaling initiated by the 9-LOX pathway.

4.3.1. Stomatal closure mechanism in *noxy76*

As *noxy76* exhibited a strong susceptibility in stomatal defense response, it was necessary to study the stomatal closure mechanism so as to get a clearer view of the defense processes mediating from the stomata.

In plants the CO_2 influx and transpirational water loss is regulated by opening and closing of stomata. Besides this, they act as protective gates to prevent the entry of pathogens (Melotto *et al*., 2006). Moreover, the significance of *LOX1*, a 9-specific LOX encoding gene, in controlling bacterial and PAMP triggered stomatal closure was demonstrated using 9-LOX synthesis mutant, *lox1* (Montillet *et al*., unpublished). As *noxy76* showed enhanced susceptibility to bacterial inoculation by spraying, it indicated that this mutant might have lost the capability to sense or to respond to bacteria on its surface and hence fails to close the stomata in response to bacteria. Furthermore, *noxy76* being a signaling mutant of the 9-LOX pathway can also behave similarly as the synthesis mutant, *lox1* in controlling stomatal closure mechanism.

4.3.1.1. Stomatal closure response to *Pseudomonas syringae* is impaired in *noxy76*

4.3.1.1.1. Response to *Pseudomonas syringae* DC3000

In wild type plants, the perception of PAMP's on the leaf surface leads to the activation of stomatal closure mechanism within 1 hr. So as to counteract this response *Pseudomonas* has evolved mechanisms to reopen the stomata. Out of those, the production of coronatine by various pathovars of *Pseudomonas* is an important aspect for evading plant defense responses and enhancing disease symptoms. Coronatine counteracts stomatal closure after *Pst* DC3000 infection and leads to re-opening of the stomata after 3 hrs post infection thereby facilitating the entry of pathogen (Melotto *et al*., 2006).

As *noxy76* was susceptible to *Pst* DC3000 on surface inoculation, the probability that it has a failure in stomatal closing mechanisms was investigated. The stomatal apertures were measured after 1 hr in leaf peals incubated in a suspension of *Pst* DC3000. The stomatal measurements revealed that the stomata was closed about 70% in response to *Pst* DC3000 in wild type, whereas the stomata of *noxy76* remained open after bacterial treatment as in non-infected leaf peals (Figure 17). By 3 hrs post infection, the stomata are reopened due to the action of coronatine in the wild type plants, while in *noxy76* the aperture width remains the same as before. Thus, an increased susceptibility of *noxy76* to *Pst* DC3000 is due to its failure in closing the stomata on the perception of the pathogen.

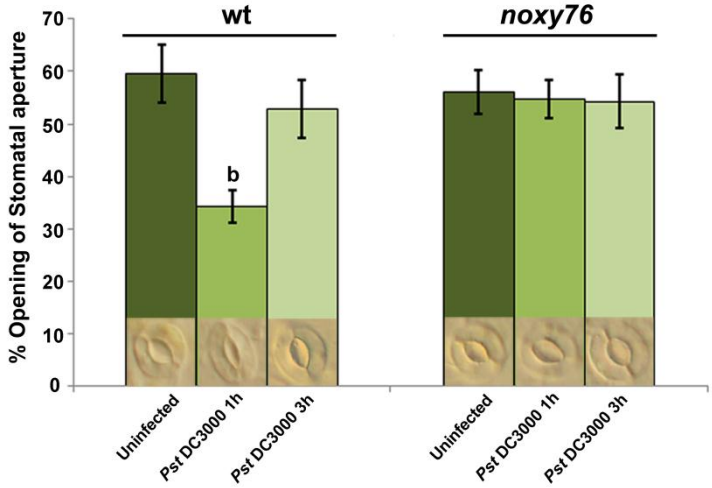

Figure 17. Examination of stomatal closure in wild type and *noxy76* plants after incubation with *Pst* DC3000

Stomatal apertures were measured in leaf peals after incubation for 1 hr and 3 hrs with *Pst* DC3000 (10^8cfu/ml). Stomatal apertures in uninfected leaf peals of wild type and *noxy76* were used as controls in these experiments (Student's t test: $^b0.001 < P < 0.01$).

4.3.1.1.2. Response to the coronatine deficient strain *Pst* DC3000 *COR*⁻ AK87

In a previous study, *ila-3*, a mutant allele of *noxy76* was found to have open stomata after 1 hr post inoculation with *Pst* DC3118, another strain of *Pseudomonas syringae* deficient in coronatine production (Zeng *et al*., 2011). In the present study, we found that the inoculation of wild type peals the coronatine deficient strain *Pst* DC3000 *COR*⁻ AK87 provoked a decrease in the stomatal aperture of about 20% and that the stomata remain closed by 20% in comparison to controls at 3 hrs after bacterial inoculation. The stomata of *noxy76* remained unresponsive to the presence of bacteria at 1hr and 3hrs, as the stomatal aperture measurements did not reveal any differences between the untreated control peals and the peals incubated in bacteria (Figure 18).

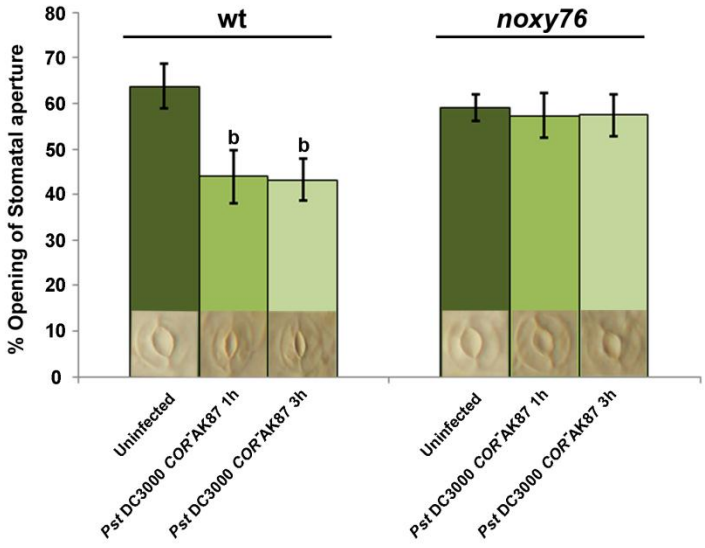

Figure 18. Examination of stomatal closure in wild type and *noxy76* plants after incubation with *Pst* DC3000 *COR*⁻ AK87

Stomatal apertures were measured in leaf peals after incubation for 1 hr and 3 hrs with *Pst* DC3000 *COR*⁻ AK87 (10^8cfu/ml). Stomatal apertures in uninfected leaf peals of wild type and *noxy76* were used as controls in these experiments (Student's t test: $^b 0.001 < P < 0.01$).

4.3.1.2. Stomatal closure response to ABA

The failure of *noxy76* to perceive bacteria and respond to its presence by closing the stomata indicated a possible failure in the signaling events leading to stomatal closure. Based on the response of *noxy76*, a study of stomatal closure mechanism in general was initiated to get a further insight into the defense susceptibility of *noxy76*.

Abscisic acid is one of the most crucial factors governing the stomatal closure mechanism. Its significance in regulating the stomatal aperture during drought and transpirational water loss

has been well documented (Schroeder *et al*., 2001). Besides this, ABA is known to have a positive role in pre-invasive defense (de-Torres *et al*., 2007).

As *noxy76* was affected in stomatal closure in response to *Pseudomonas syringae*, the possibility of this mutant to be affected in stomatal closure mechanisms mediated by ABA was analyzed. Stomatal peals were treated with ABA and the stomatal apertures were measured after 6 hrs of treatment. A decrease in the stomatal aperture of about 28% was observed in the case of wild type and in *noxy76* also. So the failure in stomatal closure in *noxy76* is independent of ABA (Figure 19).

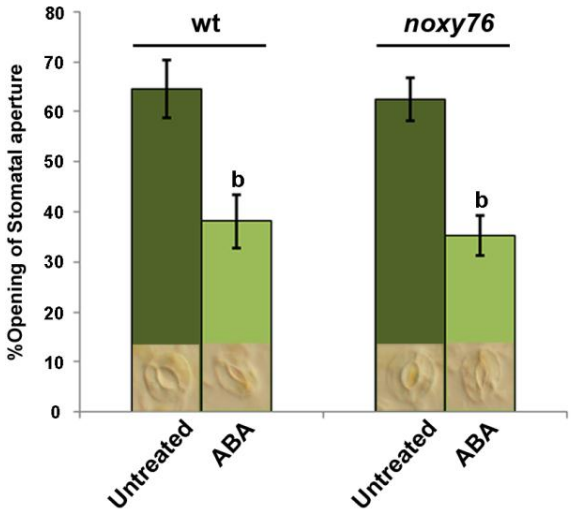

Figure 19. Examination of stomatal closure in wild type and *noxy76* plants responding to ABA application. Stomatal apertures were measured in leaf peals after incubation in stomatal buffer (untreated) and ABA (10 µM) for 6 hrs (Student's t test: $^{b}0.001 < P < 0.01$).

4.3.1.3. Stomatal closure response to oxylipins

Fatty acid hydroperoxides and reactive electrophile oxylipins containing α, β-unsaturated carbonyl structures arising due to LOX activities have been shown to induce stomatal closure (Montillet *et al*., unpublished), and the absence of 9-LOX activity led to a failure in stomatal closure in response to bacteria. This scenario was investigated in *noxy76*, a 9-LOX signaling mutant.

The stomatal apertures were measured in wild type and *noxy76* in response to 9-KOD, 9-KOT and 9-HOT. In wild type Col-0 plants a 30% reduction was observed in the stomatal aperture in response to 9-KOD and 9-KOT, whereas the stomatal apertures remained at the same levels as in control peals of the wild type plants treated with 9-HOT. In contrast to wild type plants, *noxy76*

failed to close the stomata in response to the 9-KOD and 9-KOT and also remained open on treatment with 9-HOT similar to the wild type response (Figure 20).

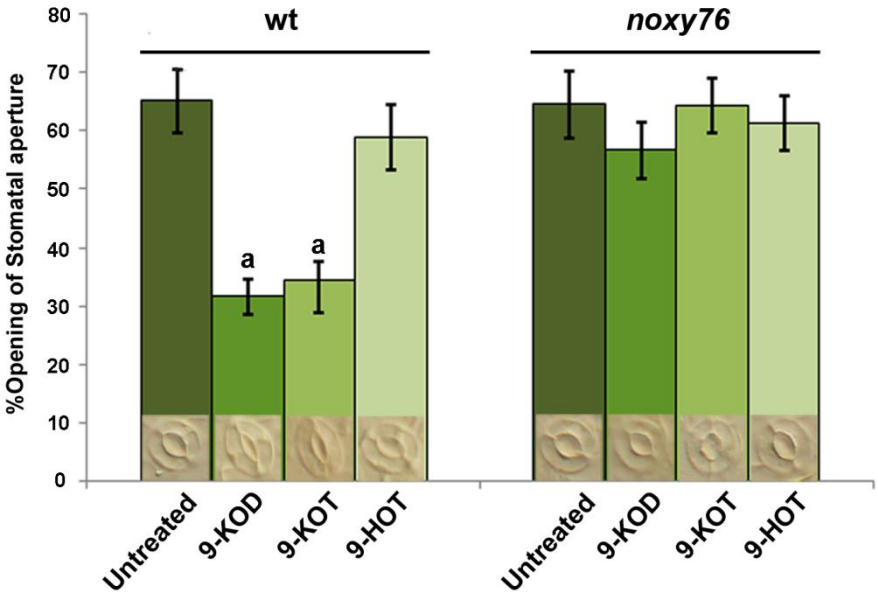

Figure 20. Examination of stomatal closure in wild type and *noxy76* plants responding to oxylipins

Stomatal apertures were measured in leaf peals 6 hrs after incubation with stomatal buffer (control), and in stomatal buffer containing 9-KOD (10 nM), 9-KOT (10 nM) or 9-HOT (10 nM) (Student's t test: $^{a}P < 0.001$).

4.3.2. Activation of 9-HOT and 9-KOT responsive genes is reduced in *noxy76*

Since *noxy76* was insensitive to the application of 9-HOT and failed to respond to 9-KOT by not closing the stomata, it was very important to carry out a molecular characterization of this mutants response to both of these oxylipins as it might also have possible alterations in the transcriptional level.

The function of *noxy76* was further investigated by studying the transcript levels of a set of genes identified through a transcriptomic analysis in response to 9-LOX oxylipin, 9-HOT (Vellosillo *et al*., 2007). The 9-HOT responsive transcripts namely, *POX* (FAD-dependent pyridine nucleotide-disulphide oxidoreductase, At5g22140), *FOX* (FAD-binding Berberine family protein with oxidoreductase activity, At1g26390) and *TOUCH3* (Calmodulin-like protein At2g41100) are induced upon treatment with both, 9-HOT and 9-KOT. The accumulation of the above mentioned transcripts was analyzed in wild type and *noxy76* root samples treated with 9-HOT and 9-KOT. The

levels of the three transcripts under study were clearly reduced in *noxy76* in comparison to wild type plants (Figure 21). The reduced accumulation of transcripts further highlights the insensitivity of *noxy76* to 9-HOT and 9-KOT and the importance of *noxy76* mutant in studying the 9-LOX pathway.

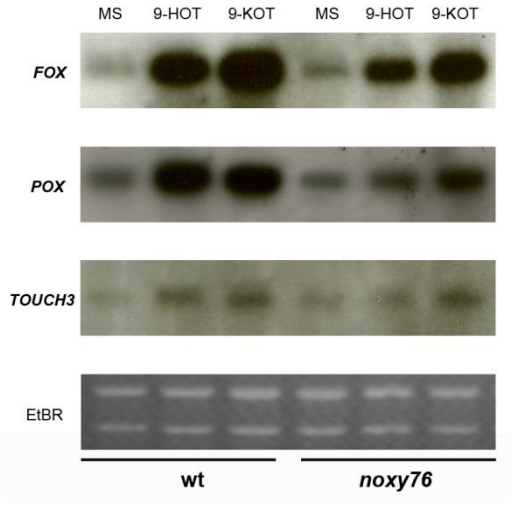

Figure 21. Expression of 9-HOT inducible marker genes in roots of wild type and *noxy76* plants

RNA was extracted from roots of 12-day-old seedlings after 9-HOT (25 µM) and 9-KOT (25 µM) application. RNA from MS treated roots was used as a control in these experiments. Blots were hybridized to riboprobes for genes encoding for *POX*, *FOX* and *TOUCH3*. Shown are representative examples of results obtained with RNA from three independent experiments.

4.3.3. Response of *noxy76* to oxidative stress

Previous studies in the laboratory have shown the generation of Reactive Oxygen Species (ROS) in the waving region of roots treated with 9-HOT (Vellosillo *et al*., 2007) and that this stress response can be quenched by trolox. Trolox is a lipophilic anti-oxidant and singlet oxygen quencher. This suggested that there exist an important role for oxidative stress in 9-HOT signaling (López *et al*., 2011). Furthermore, the singlet oxygen quenching property of trolox implicated the importance of 9-HOT signaling in response to singlet oxygen. Besides the significance of ROS in 9-HOT signaling, it also plays a crucial role in plant defense responses during a hypersensitive reaction. Hence it was pertinent to test the effect of oxidative stress generating compounds on the growth of *noxy76*, as it will enable a more detailed study of the defense responses signaled by distinct ROS.

4.3.3.1. Response to hydrogen peroxide and superoxide

The effect of hydrogen peroxide on wild type plants and *noxy76* was studied by germinating their seeds in a medium containing 2 mM hydrogen peroxide. An overall reduction in the growth of the plants was observed across both genotypes. In comparison to wild type plants grown in MS control, the plants grown in hydrogen peroxide exhibited smaller leaves at 14 days (Figure 22).

This growth defect was also observed in the mutant *noxy76*. No clear differences were observed amongst the wild type and *noxy76* seedlings, so it can be concluded that *noxy76* responds similarly to the wild type in the presence of hydrogen peroxide.

The response of *noxy76* to superoxide was studied by germinating in a medium containing paraquat, a generator of superoxide in the chloroplast. Similar to the treatment with hydrogen peroxide, paraquat also induced a reduction in overall growth amongst both genotypes after 14 days. Besides the reduction in growth no specific differences were observed between the wild type Col-0 and the mutant *noxy76* seedlings grown in paraquat (Figure 22).

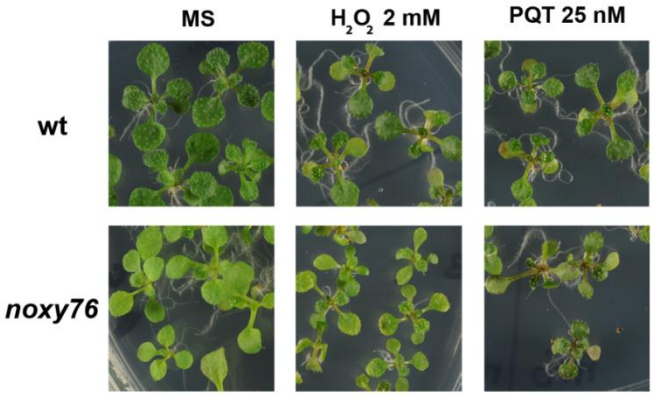

Figure 22. Phenotype analysis of wild type and *noxy76* plants responding to hydrogen peroxide and superoxide

Phenotypes of seedlings grown for 14 days in MS control and MS medium containing hydrogen peroxide (2 mM) and superoxide generator Paraquat (25 nM).

Since wild type plants and *noxy76* behaved in a similar way upon germination in hydrogen peroxide and paraquat, it can be concluded that *noxy76* is not affected in the signaling responses initiated by these types of ROS.

4.3.3.2. Response to singlet oxygen

Singlet oxygen is the other form of ROS whose role in plant defense response has been recently unraveled. Singlet oxygen is formed from molecular oxygen under excitation by high light. Furthermore, due to its highly reactive nature, singlet oxygen can initiate peroxidation of lipids (Triantaphylides and Havaux, 2009).

To begin with, *noxy*76 along with wild type Col-0 seeds were germinated in a medium containing Rose Bengal, a generator of singlet oxygen. Phenotypic changes were scored after 14 days of germination in medium containing Rose Bengal. Three distinct phenotypes scored as I, II and III (Figure 23A and 23B) were observed, based on the color and growth of the seedlings.

- **Phenotype I** (corresponding to green in Figure 23B) included seedlings that exhibited partial reduction in growth with the seedlings remaining green in color in comparison to the growth in control MS.
- **Phenotype II** (corresponding to yellow in Figure 23B) included seedlings which were bleached and yellow in color with reduced growth.
- **Phenotype III** (corresponding to dwarfed in Figure 23B) included seedlings that showed severe growth retardation and were dwarfed.

In the case of wild type Col-0 plants, 95% of the seedlings which were quantified exhibited phenotype I, whereas significant changes were observed in *noxy76*. About 64% of the seedlings exhibited phenotype I, a decrease of about 30% in comparison to wild type seedlings. Furthermore, 21% of the seedlings were bleached with yellow colored leaves and reduced growth corresponding to phenotype II and 15% of the seedlings were dwarfed corresponding to phenotype III. In the case of wild type plants only 1% and 4% of the seedlings exhibited phenotype II and phenotype III, respectively. This difference in the response of the wild type and *noxy76* to singlet oxygen revealed the enhanced susceptibility of *noxy76* to the application of singlet oxygen as compared with wild type plants.

As a clear level of susceptibility was established in *noxy76* in response to singlet oxygen, the molecular patterns leading to its susceptibility was investigated. The 9-LOX responsive transcripts namely *ABC* (encodes an ABC transporter, At1g15520), *FOX*, *POX* and *LOX1* (encoding a 9-lipoxygenase) were also induced by singlet oxygen. For transcript analysis, 12 day old seedlings were covered with Rose Bengal and samples collected after different time points. In the case of wild type plants, a clear induction of the above mentioned transcripts was observed by 9 hrs after treatment, while in *noxy76* the induction of those genes were significantly reduced (Figure 23C). The transcription of *ABC*, a widely used marker gene for stress responses was completely abolished in *noxy76* (Figure 23C), while the levels of other transcripts were significantly diminished with a stronger reduction in the case of *LOX1.* The decreased levels of these transcripts confirm the susceptibility of *noxy76* to singlet oxygen and the involvement of 9-LOX pathway in signaling responses to singlet oxygen.

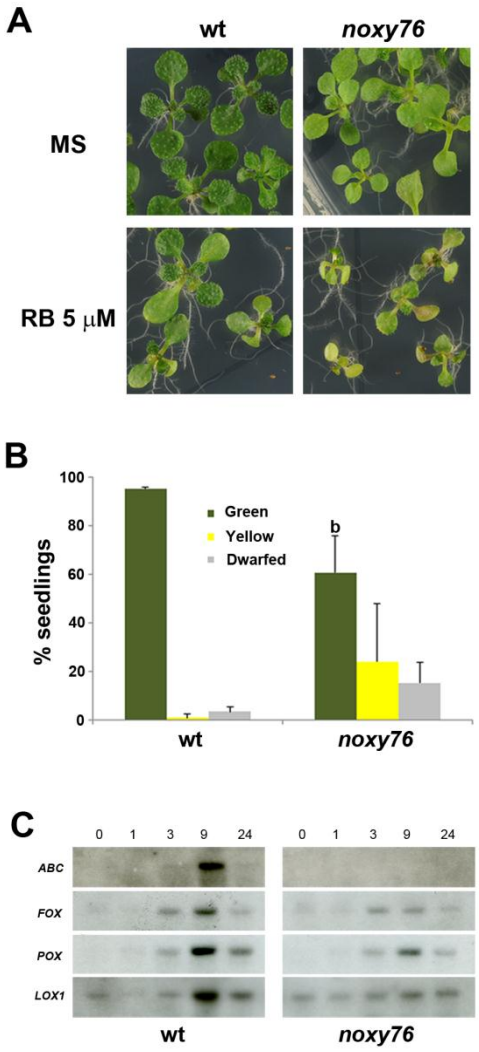

Figure 23. Analysis of wild type and *noxy76* plants responding to the singlet oxygen generator Rose Bengal

(A) Phenotype of seedlings grown for 14 days in MS control (Top row) and MS medium containing RB (5 µM) (Bottom row).

(B) Phenotypic alterations were scored on a three-point scale (I, II and III), according to the severity of the symptoms. The percentages of seedlings showing these phenotypes in RB-containing medium are shown. Values are means and standard errors from three independent experiments. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's t test: $^{b}0.001 < P < 0.01$).

(C) Analysis of singlet oxygen marker genes in seedlings of wild type Col-0 and *noxy76* plants treated with Rose Bengal 10 µM. Blots were hybridized to riboprobes for genes encoding for *ABC, FOX, POX*, and *LOX1*. Shown are representative examples of results obtained with RNA from three independent experiments.

4.3.4. Response to nitric oxide

Besides the examination of responses to various ROS, the effect of nitric oxide (NO) was also studied in *noxy76*. NO is a gaseous reactive nitrogen species which is an important signaling molecule in plant defense responses. Its lipophilic nature enables faster diffusion through the membrane and it is known to induce pathogen related genes during pathogen infection (Hong *et al*., 2008). In addition to the apoplastic defense, NO production is necessary for ABA mediated stomatal closure and hence has an important role in stomatal defense responses. Moreover, NO in conjunction with various ROS play a crucial role in enhancing resistance against pathogen infection. Hence, it became important to study the responses *noxy76* to exogenous application of NO similar to the various ROS previously analyzed.

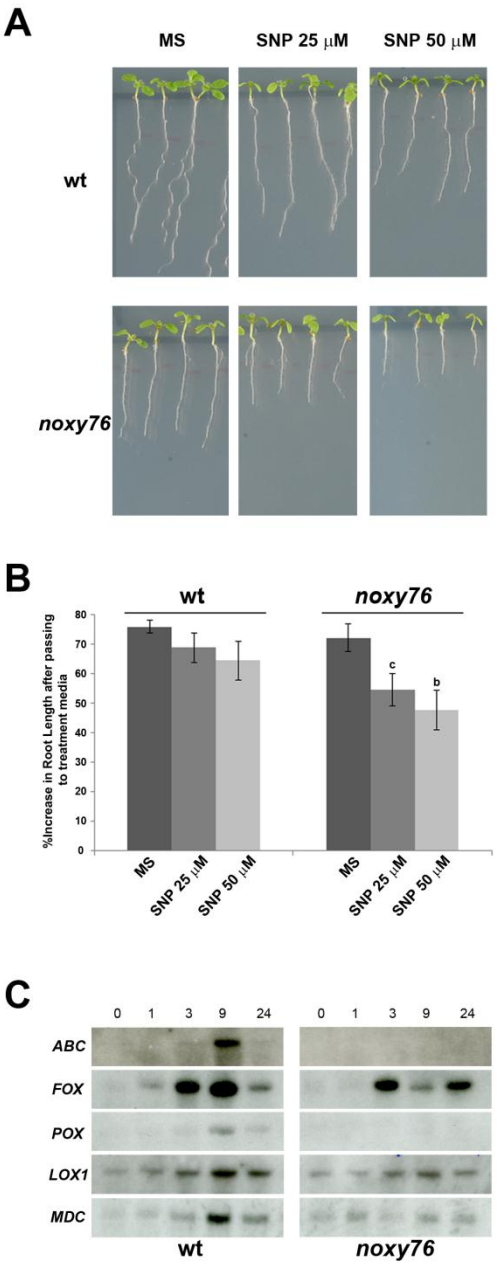

Figure 24. Analyses of wild type and *noxy76* plants responding to nitric oxide donor sodium nitroprusside

(A) Dose response effect of SNP on the growth of wild type Col-0 and *noxy76*. Phenotype of 7-day-old plants grown in MS for 3 days and then transferred to control MS medium and MS medium supplemented with SNP (25 and 50 μM).

(B) Root length measurements were done using ImajeJ software. Roots were measured on the 4th day after passing to control and SNP. Increase in root length after moving seedlings to MS and SNP-containing medium is measured and expressed as percentage of growth with respect to the final length of roots at the end of the experiments (Student's t test: $^{b}0.001 < P < 0.01$, $^{c}0.01 < P < 0.05$)

(C) Analysis of nitric oxide marker genes in wild type and *noxy76* plants after application of SNP. RNA was extracted from 12-day-old seedlings at different intervals after SNP (100 μM) application. Blots were hybridized to riboprobes for genes encoding for *ABC, FOX, POX*, and *LOX1*. and *MDC*. Shown are representative examples of results obtained with

Sodium nitroprusside (SNP) was used as a NO donor in our studies. SNP was able to induce partial root growth arrest in wild type seedlings and *noxy76* seedlings with the effect stronger at a concentration of 50 μM (Figure 24A), even though reduction was also observed at 25 μM.

Since the wild type Col-0 and *noxy76* grew at different rates, with *noxy76* having shorter roots, a quantification of the root lengths was performed so as to gauge any minor differences that might be present in their responses to SNP. Root length measurements revealed a significant

difference in the responses of wild type and *noxy76* seedlings. SNP was able to induce a stronger root growth reduction in *noxy76* seedlings than in wild type Col-0 seedlings (Figure 24B). This result suggests that *noxy76* is more susceptible than the wild type Col-0 to the exogenous application of NO.

The susceptibility of *noxy76* to NO was also manifested at the transcriptional level. Transcript analysis was carried out on RNA samples obtained from 12 day old seedlings of wild type and *noxy76* treated with SNP. A very clear difference was observed when analysis of the levels of 9-LOX responsive transcripts (*ABC, FOX, POX, LOX1* and *MDC*) was done. The transcription of *ABC* and *POX* was completely abolished while a clear reduction in the accumulation of the other transcripts was also observed. This transcriptional analysis revealed that *noxy76* mutant failed to respond to NO and was not able to induce stress responsive transcripts similar to the levels in wild type plants after exogenous application of NO (Figure 24C) and hence the enhanced susceptibility to exogenous nitric oxide application.

4.3.5. *noxy76* is affected in the production of nitric oxide induced by *Pseudomonas syringae*

Out of the various factors which are involved in the stomatal closure mechanism, the production of NO is an important response which precedes stomatal closure (Desikan *et al.,* 2004). Moreover, previous studies have indicated that the production of NO in the stomata leads to stomatal closure during flg22 treatment (Melotto *et al*., 2006), thereby playing a positive role in stress responses.

The results from the characterization of *noxy76* revealed the failure of these plants in closing the stomata after bacterial inoculation as well as the defect in NO signaling. Hence a further examination of nitric oxide production in the stomata of *noxy76* was undertaken.

The lower epidermal peals from wild type and *noxy76* were treated with *Pst* DC3000 and *Pst* DC3000 *COR*$^{-}$ AK87 and stained for NO after 1 hr of treatment. A clear difference was observed among wild type and *noxy76* peals. Wild type stomata showed fluorescence as a result of the reaction between DAF2 and NO produced during bacterial inoculation (Figure 25), while the *noxy76* stomata did not exhibit the fluorescence thereby indicating the failure of *noxy76* to produce NO during bacterial inoculation.

This result indicates that the failure in the stomatal closure mechanism of *noxy76* in response to *Pseudomonas* is due to the impairment in NO production in this mutant which ultimately leads to increased bacterial entry, thereby rendering this mutant more susceptible to both strains of *Pseudomonas*.

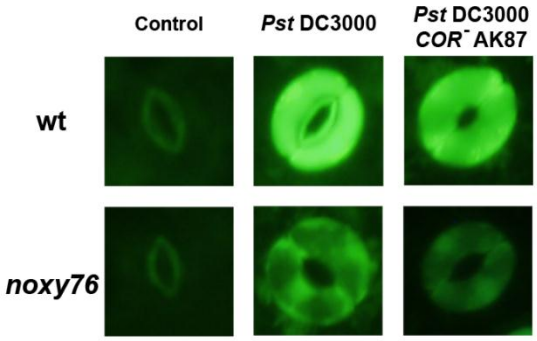

Figure 25. Analysis of NO production in guard cells during bacterial infection

In situ visualization of NO production by DAF-2DA staining of the guard cells in peals of wild type Col-0 and *noxy76* plants after treatment for 1hr with stomatal buffer (left column), *Pst* DC3000 (10^8cfu/ml) (middle column) and *Pst* DC3000 *COR*- AK87 (10^8cfu/ml) (right column).

Discussion

5. DISCUSSION

One of the responses of plants to pathogen infection is the generation of a class of lipid derivatives called as oxylipins which are known to play important roles in physiological processes and defense responses (Savchenko *et al.,* 2010; López *et al*., 2008 and 2011). These compounds are mainly synthesized through the action of α-dioxygenases (α-DOX) and lipoxygenases (9-LOX and 13-LOX) which incorporate molecular oxygen in the 2nd, 9th and 13th position of the fatty acid molecule, respectively (Hamberg *et al*., 1999; Feussner and Wasternack, 2002). Extensive research has been dedicated to the study of JA, an oxylipin derived from 13-LOX pathway and its role in plant development and defense responses has been well elucidated (Browse 2009). Contrary to the 13-LOX pathway, the study of oxylipins from the 9-LOX and α-DOX pathways have received little attention, although their importance in plant development and defense responses is starting to be elucidated (Sanz *et al*., 1998; De Leon *et al*., 2002; Hamberg *et al*., 2003; Andersson *et al*., 2006; Vellosillo *et al*., 2007; Bannenberg *et al*., 2009; López *et al*., 2011; Vicente *et al*., 2012).

Recent studies in our laboratory have highlighted the importance of 9-LOX activity in defense against hemibiotrophic bacteria (López *et al*., 2011; Vicente *et al*., 2012). These studies which revolved around the two 9-LOX enzymes of *Arabidopsis*, LOX1 and LOX5, and the mutants *lox1* and *lox5* devoid of 9-LOX activity demonstrated the role of this oxylipin pathway in plant defense mainly by controlling the oxidative stress accompanying the response of *Arabidopsis thaliana* to infection.

A forward genetic approach was utilized to further characterize the 9-LOX pathway and mutants insensitive to the application of 9-HOT were isolated. These mutants failing to perceive 9-HOT represent a valuable tool to study the 9-HOT signaling and three of them, namely *noxy3*, *noxy72* and *noxy76* were chosen for characterization.

5.1. Callose and lignin deposition is impaired in *noxy* mutants

One of the responses of wild type plants to application of 9-HOT is the deposition of callose, a high molecular weight β-glucan polymer which serves as a physical barrier to prevent the entry of pathogens into the plant (Brown *et al*., 1998). The accumulation of callose after 9-HOT treatment highlights the role of 9-LOX pathway in activating basal defense. Accordingly, the fact that the *noxy*

mutants analyzed in this study were impaired in the deposition of callose after 9-HOT application (Figure 1) indicated that the defense responses in these mutants might be impaired.

In addition to 9-HOT, previous studies in our laboratory have shown that some *noxy* mutants were partially insensitive to the application of isoxaben, an herbicide which inhibits cellulose synthase leading to root growth arrest, root thickening and lignification (Desprez *et al*., 2002). Lignin is a phenolic polymer resulting from the oxidative polymerization of monolignols and is a major component of secondary cell wall (Boerjan *et al*., 2003). Lignin along with suberin is the constituent of the wound periderm acting as a mechanical barrier during plant pathogen interaction (Schreiber *et al*., 1999). In this manner plants with higher lignin content are more resistant to microbes as lignification of cell wall is an important aspect of plant defense against pathogens and insect herbivory (Kawasaki *et al*., 2006). Our results from studying the response of *noxy* mutants to isoxaben indicated that *noxy3*, *noxy72* and *noxy76* fail to induce lignification (Figure 2) thus supporting the notion that these *noxy* mutants might probably be altered in their defense responses against pathogens.

5.2. *noxy3* showed enhanced resistance against the coronatine deficient strain *Pst* DC3000 *COR*$^-$ AK87

We have employed *Pseudomonas syringae* as a model pathogen to study the defense response of the *noxy* mutants and to dissect the resistance patterns mediated by the 9-LOX pathway. Based on the infection mechanisms used by these pathogens, the introduction of bacteria on to the plant was executed in two ways, namely by infiltration and spray inoculation. The former is an efficient way to study apoplastic defense in the intercellular spaces, while the latter involves the entry of bacteria through the natural openings of the plant, predominantly through the stomata.

As expected in these studies we found that irrespective of the mode of inoculation, symptom development and bacterial growth in wild type plants reached similar levels when infected with *Pst* DC3000, whereas this was not the case with the coronatine deficient strain *Pst* DC3000 *COR*$^-$ AK87, for which the symptoms and bacterial growth were clearly lower upon surface inoculation than after infiltration into the apoplast. In the same manner the response of *noxy3* to bacterial infection did not vary from wild type plants in the case that bacteria were applied by infiltration. Accordingly, no significant changes were observed during staining for hydrogen peroxide and cell death (Figure 3), thus indicating that the *noxy3* mutation does not affect apoplastic defense.

Moreover no significant differences between wild type plants and *noxy3* mutants were observed in their response to surface inoculation with *Pst* DC3000, although a trend to a reduced bacterial growth was observed in *noxy3* plants. The differences between wild type and *noxy3* mutants became apparent when the *Pst* DC3000 *COR*$^{-}$ AK87 was spray inoculated. The partial chlorosis which was visible in wild type plants upon spray inoculation was reduced in the case of *noxy3* and the quantification of bacterial growth revealed a significant (5 fold) decrease in *noxy3* as compared with the wild type plants (Figure 7). This result reveals the enhanced resistance of *noxy3* against *Pst* DC3000 *COR*$^{-}$ AK87 when delivered through the stomata and thus it shows the enhanced pre-invasive immunity of *noxy3*.

Coronatine is a polyketide phytotoxin which is known to favor bacterial infection by inducing stomatal reopening and suppressing SA mediated defense responses (Mittal and Davis, 1995; Melotto *et al*., 2006; Zhao *et al*., 2003). The action of coronatine by mimicking JA, contributes to activate JA-mediated responses and antagonizes the induction of SA defense during surface inoculation with the virulent strain of *Pseudomonas*, thereby enhancing its virulence potential (Weiler *et* al., 1994; Melotto *et al*., 2008). Since the coronatine deficient strain *Pst* DC3000 *COR*$^{-}$ AK87, does not have the capacity to reopen the stomata, it is likely that the results showing that the *noxy3* mutant is resistant to spray inoculation by this bacterial strain, indicated that the stomatal closure mechanism of *noxy3* is activated above levels in wild type. Moreover, the fact that *Pst* DC3000 reached similar growth when infecting wild type and *noxy3* plants indicated that the defect found in *noxy3* mutants can be overcome by coronatine.

Based on the faster and increased capacity of *noxy3* to close stomata in response to *Pst* DC3000 *COR*$^{-}$ AK87 it is possible that the SA production or the SA-mediated defenses could be activated above levels of wild type plants in *noxy3* mutants. Even though this hypothesis cannot be ruled out presently, this possibility seems unlikely because overproduction of SA or its enhanced signaling would be accompanied by enhanced apoplastic defense, in which case *noxy3* behaved like wild type.

As an alternative explanation, the fact that *noxy3* mutants showed enhanced pre-invasive defense when infected by *Pst* DC3000 *COR*$^{-}$ AK87 could be correlated with our preliminary results which showed that that *noxy3* is hypersensitive to the exogenous application of ABA; a plant hormone known to induce stomatal closure in response to a wide variety of abiotic and biotic stresses (Schroeder *et al*., 2001). Thus enhanced production or signaling of ABA could favor stomatal closure and prevent bacterial entry. However, this possibility would not explain the

results obtained with *Pst* DC3000 for which *noxy3* mutants showed a trend to reduced bacterial entrance but the difference with wild type remained non-significant. Moreover this interpretation will not explain why *noxy3* showed wild type apoplastic defense as enhanced activation of ABA signaling should antagonize SA-mediated defense and thus apoplastic resistance.

Finally, the production of nitric oxide is an important component in ABA-mediated (Neill *et al*., 2002; Desikan *et al*., 2004). Therefore, an investigation into the NO accumulation in stomata and an analysis of stress responsive transcripts will throw further light on the resistance mechanism in *noxy3*.

5.3. *noxy72* exhibits enhanced apoplastic defense responses

The characterization of responses to *Pseudomonas syringae* revealed that *noxy72* exhibited enhanced apoplastic defense to the virulent strain which was clearly visible in the reduced bacterial growth and symptom development (Figure 4A). In most of the cases, plant resistance to biotrophic pathogen is accompanied by localized cell death or a hypersensitive reaction which includes the generation of reactive oxygen species and callose deposition to prevent further colonization of the pathogen (Apel and Hirt, 2004; Gechev *et al*., 2006; Triantaphylidés and Havaux, 2009; Nanda *et al*., 2010; Jacobs *et al*., 2003; Nishimura *et al*., 2003; Boller and Felix, 2009). On the contrary, the above mentioned parameters, cell death and H_2O_2 accumulation, were reduced in *noxy72*, but still this mutant harbored less growth of the virulent strain in comparison to wild type plants. The fact that *noxy72* showed reduced H_2O_2 accumulation was indicative of an alteration in ROS homeostasis. Therefore, it is possible that the increased resistance might be conferred by an increased production of other ROS like superoxide, singlet oxygen or nitric oxide. Additionally the resistance in apoplastic responses can also be implicated on a higher induction of SA biosynthetic and responsive genes. Further investigation of the molecular patterns is necessary to validate the mechanisms underlying resistance in *noxy72*.

5.4. Role of *NOXY3* and *NOXY72* in plant defense

As discussed above, our previous results in the study of the 9-LOXs enzymes revealed the positive role of this oxylipin pathway in the defense of plants against infection of pathogenic *Pseudomonas* bacteria. However, in contrast to our expectations, the results from the characterization of the 9-HOT insensitive mutants, *noxy3* and *noxy72,* revealed that both mutations

exhibited enhanced pre-invasive and apoplastic defenses, respectively, and hence that *NOXY3* and *NOXY72* act as negative regulators of plant defense.

The map-based positional cloning of *noxy3* has enabled the localization of this mutation in the locus At5g26860, which encodes the LON1 protease that is localized in the mitochondria. Similar to *noxy3*, the map-based positional cloning of *noxy72* enabled us to localize this mutation in the locus At5g57300, encoding a mitochondrial S-adenosyl-L-methionine-dependent methyltransferases that is involved in the synthesis of ubiquinone. The mapping of *noxy3* and *noxy72* to two proteins that are sorted to mitochondria goes with the trend observed in our laboratory, as out of 10 *noxy* mutants identified to date, 6 of them encoded proteins that are localized in the mitochondria. In this context, the fact that a high proportion of the 9-HOT insensitive mutants identified encoded mitochondrial proteins, together with additional results showing that 9-HOT impact on the mitochondria to cause membrane depolarization, revealed the importance of these organelles on the signaling processes activated by this oxylipin.

Extensive studies in bacteria have revealed that LON proteases are stress responsive proteases which are expressed during a wide variety of conditions producing oxidative damage (Ondrovicova *et al*., 2005). In a similar manner mitochondrial LON protease has been implicated in regulating cellular redox homeostasis in mammals were these proteases act by degrading oxidatively modified proteins under hypoxia or endoplasmic reticulum stress conditions (Hori *et al*., 2002). In line with the proposed role of LON proteases, we note that the application of 9-HOT enhances the production of superoxide ion and activates the expression of oxidative stress-related genes (Vellosillo *et al*., 2007; López *et al*., 2011). This brings us to the point wherein, NOXY3 might play a role in protein quality control in the mitochondria during stress conditions. In this context NOXY3 might play an active role in degrading oxidatively modified proteins formed due to the oxidative properties of 9-HOT and thereby prevent accumulation of deleterious complexes within the mitochondria, thus helping in maintaining proper mitochondrial function.

Similar to NOXY3, the role of NOXY72 proteins in the 9-HOT signaling might be related with the oxidative stress generated after the application of this oxylipin. The S-adenosyl-L-methionine-dependent methyltransferases encoded by *NOXY72* is a coenzyme Q5/UbiE which is involved in the ubiquinone biosynthesis. Ubiquinone has a central role in plant mitochondrial electron transport wherein it is reduced to ubiquinol by the complexes I and II. Ubiquinol in turn can be oxidized by the cytochrome pathway or the alternative oxidase pathway (Popov *et al*., 2001). In addition to its role as a substrate, ubiquinol might act as a ROS scavenger, in particular superoxide, which is

produced during mitochondrial electron transfer (Rich and Bonner 1978). Thus NOXY72 might be involved in controlling the oxidative stress responses during 9-HOT signaling and mitochondrial electron transport.

The function of the NOXY3 and NOXY72 identified are in agreement with our previous results showing role of the 9-LOX pathway in controlling ROS homeostasis (López *et al*., 2011). Moreover, these findings support the action of 9-HOT in inducing ROS generation after its application and with the fact that the *noxy* mutants characterized here were selected on the basis of their insensitivity to this oxylipin. Studies by numerous laboratories and our own results have demonstrated that the ROS production and ROS signaling pathways interact through complex regulatory networks in which the reduction of specific type of ROS might cause enhanced production and activation of signaling pathways responding to other compounds (Laloi *et al.,* 2007; López *et al*., 2011). Although further studies will be needed to determine the effect of the *noxy3* and *noxy72* mutations in ROS production and signaling, it is likely that the enhanced resistance observed could be the consequence of an alteration in the production of ROS and in the signaling mechanisms mediating the responses to these reactive compounds. This idea was strongly supported by the fact that NOXY3 and NOXY72 proteins are localized in the mitochondria which is known to be a major source of ROS in plants.

Numerous lines of evidence have recently highlighted the importance of mitochondria and mitochondrial functions in the regulation of host innate immune signaling. Compelling data have introduced the concept that mitochondria in addition to their well-known role in cell metabolic processes and cell death, are crucial assembly platforms for innate immune signals and that innate immunity and basic mitochondrial functions are integrated (Arnoult *et al*., 2011). An attractive hypothesis starting to be tested is that the convergence of multiple innate immune responses in these organelles would allow for a tight functional integration of host defense and metabolic processes. In this manner, the activation of certain immune responses through an energetic checkpoint will ensure that efficient activation of defense pathways is properly balanced and turned out in proper cells.

Even though knowledge on the role of mitochondria in plant innate immunity is still poor, the importance of these organelles in plant defense is emerging. Thus, a number of studies have revealed that the disruptions of the mitochondrial function provoke significant variation in the outcome of a plant pathogen interaction and that application of specific compounds acting on the mitochondria affects the expression of defense related genes (Gleason *et al*., 2011; Schwarzlander

et al., 2012). As discussed above, the studies in our lab have shown that a high proportion of *NOXY* genes encoded mitochondrial proteins and that their mutations provoked significant changes in their response to *Pseudomonas* infection. Of interest, out of the 6 *noxy* mutants encoding mitochondrial proteins three have been found to be resistant to bacterial inoculation, including *noxy3* and *noxy72*, while 3 of them showed enhanced susceptibility as compared with wild type. Therefore, in line with our discussion, the fact that these *noxy* mutants showed an alteration in their ability to cope with pathogen infection support the role of mitochondria in these response and indicated that the *noxy* mutants will represent a valuable tool to gain a further insight in the role of these organelles in plant defense as well an in the signaling pathways controlling mitochondrial-nuclei communication.

5.5. *noxy76* is defective in stomatal and apoplastic defense responses

The characterization of responses to *Pseudomonas* revealed that *noxy76* was susceptible when infiltrated directly into the apoplastic space (Figure 5A). The susceptibility was exhibited against both the virulent and the avirulent strain of *Pseudomonas* suggesting a severe compromise in the activation of` defense responses in this mutant. Furthermore, in the response to the avirulent strain the susceptibility was accompanied by an increased accumulation of hydrogen peroxide, while the opposite was observed during the response to the virulent strain that, in addition was accompanied by decreased cell death (Figure 5B). The altered accumulation of hydrogen peroxide observed in *noxy76* plants takes us back to the situation with other *noxy* mutants which exhibited differential accumulation of ROS and hence the possibility that *noxy76* exhibited an alteration of ROS homeostasis. Indeed, the results obtained with *noxy76* parallel to those obtained previously during the characterization of *lox1 lox5* and *noxy22* (López *et al*., 2011), in which enhanced susceptibility to *Pst* DC3000 was accompanied by a similar modification in ROS accumulation and a failure in activating cell death.

In addition to a role in apoplastic defense, the fact that surface inoculation with *Pseudomonas* resulted in a susceptibility of higher magnitude in *noxy76* than in wild type plants, indicated that the *noxy76* mutant was impaired in activating pre-invasive defenses (Figure 8). This higher susceptibility suggested a probable failure on the part of *noxy76* to sense or respond to the presence of bacteria, which in the wild type leads to stomatal closure to prevent bacterial entry. In support of this idea, we found that *noxy76* responded in a similar way to the presence of both the virulent and coronatine deficient bacterial strain which indicated that the mutation in *NOXY76* is

able to rescue the virulence phenotype of this bacterial mutant and thus, that the *noxy76* mutation facilitates bacterial entrance through the stomata.

As a further conclusion, the characterization of responses of *noxy76* to *Pseudomonas* syringae suggested the role of NOXY76 as a positive regulator of plant defense that functions at different layers to protect plants against distinct bacterial strains.

5.6. *NOXY76* is necessary for stomatal closure to *Pseudomonas syringae* but not for ABA mediated stomatal closure

The higher bacterial density achieved by *Pseudomonas* after surface inoculation of *noxy76* mutants indicated the failure of this mutant in activating pre-invasive defense. This idea was supported by results from examining stomatal aperture in wild type and *noxy76* plants. Thus, the fact that wild type plants reduced stomatal apertures by almost 25% and 22% at 1hr after infection with the virulent and the coronatine deficient strains of *Pseudomonas*, respectively, whereas the *noxy76* mutant failed to close the stomata after spray inoculation of both bacterial strains (Figures 16 and 17), demonstrated the defect of *noxy76* to regulate stomatal closure. This assumption was sustained by the results showing that the stomata of *noxy76* remained open 3 hours after bacterial inoculation and thus, that stomatal closure was not delayed with respect to control plants but that indeed *noxy76* plants were impaired stomatal closure during bacterial infection.

Studies on the mechanisms mediating stomatal aperture have demonstrated the role of ABA as an essential player in stomatal closure mechanism during abiotic stress responses. In response to stress conditions the increased production of ABA leads to an increase in the cytoplasmic Ca^{2+} levels which is accompanied by an efflux of anions through anion channels followed by reduction of turgor pressure that result in stomatal closure (Siegel *et al*., 2009; Hedrich *et al*., 1990; Linder and Raschke, 1992; Schroeder and Hedrich, 1989). In addition to a role in abiotic stress the importance of ABA during pre-invasive defense was previously documented using the ABA deficient *aba3-1* mutant, which was found to be susceptible to the coronatine deficient *Pseudomonas* strain due to its failure in triggering stomata closure upon bacterial inoculation. Importantly, we found that both wild type and *noxy76* respond in a similar manner to ABA by triggering stomata closure and therefore that the failure of *noxy76* was independent of ABA.

5.7. *NOXY76* is a component of 9-LOX mediated stomatal closure

The involvement of 9-LOX pathway in stomatal defense responses was highlighted by the unpublished data of Montillet and colleagues through the findings that (i) *LOX1* is expressed in the guard cells of stomata, (ii) the absence of 9-LOX activity compromised the activation of pre-invasive defense and (iii) Reactive Electrophilic Species-Oxylipins such as 9-KODE and 9-KOTE generated through the 9-LOX pathway were able to induce stomatal closure at nanomolar concentrations, while their corresponding alcoholic non-electrophilic oxylipin (9-HOT) did not. Based on these results it was possible that the defect of *noxy76* could be associated with the failure of this mutant in 9-LOX signaling. In support of this conclusion we found that *noxy76* failed to respond to the application of 9-KOT and 9-KOD by closing the stomata (Figure 20) and thus, that this mutant was impaired in the response to these oxylipins. The results of these analyses support the role of the 9-LOX pathway at pre-invasive defense as well as the participation of the NOXY76 protein in this pathway.

5.8. *NOXY76* encodes a HEAT repeat protein, ILITYHIA

Map-based cloning identified the *noxy76* mutation in locus At1g64790 encoding *ILITYHIA* (*ILA*), a gene of unknown function that was identified in a search for genes involved in female gametophyte and embryo sac development (Johnston *et al*., 2007). The mutation in *noxy76* was a G to A transition at the last nucleotide of the 22nd intron which changes the sequence at the splice site thereby leading to a failure in its recognition by the spliceosome machinery (Figure 14). This phenomenon was confirmed through semi quantitative RT-PCR that showed the retention of the 22nd intron in *noxy76* and thus, that translation of *noxy76* transcripts would lead to the formation of a protein with additional amino acids that could ultimately compromise its function (Figure 15). The growth of *noxy76* revealed some phenotypic alterations such as the serrate shape and a pale green coloration of the leaves, which might be indicative of a decreased production of chlorophyll during the first stages of plant development. Moreover, the comparison of *noxy76* with other *ila* alleles, in which the lack of ILA function caused seed abortion and sterility (Johnston *et al.,* 2007; Monaghan *et al.,* 2010) indicated that the *noxy76* mutation does not abolish protein function but that *ila* retained a partial level activity.

Sequence analyses of the ILA protein revealed a significant level of homology with GCN1 (General Control Nonderepressible1), a regulator of protein translation controlling amino acid

starvation (Sattlegger and Hinnebusch, 2005). The characterization of this response in yeast and humans has demonstrated the ability of GCN1 to sense and interact with the uncharged tRNAs accumulating during amino acid starvation to initiate a signaling cascade that ultimately induce the expression of the amino acid biosynthetic genes. In this cascade, GCN1 works by interacting with GCN2, a protein kinase which upon activation phosphorylates the translation initiating factor eIF2α leading to its inactivation. The inactivation of eIF2α blocks general translation but facilitate the translation of GCN4, a transcription factor which in turn activates the expression of amino acid synthesis genes. In support of the role of GCN1 as regulator of protein translation, the results of Sattlegger and Hinnebusch (2005) demonstrate that the mutation in GCN1 led to decreased phosphorylation of eIF2α and hence that GCN1 has a crucial role in regulating translation of mRNA. Like in yeast, studies in *Arabidopsis* revealed that mutation of GCN2 reduced the phosphorylation of the initiating factor eIF2α (Zhang *et al.,* 2008), thus suggesting a similar role for the *Arabidopsis thaliana* GCN2 as well as for the GCN1 homolog ILA. Besides further studies will be needed to determine the role of GCN1 in *Arabidopsis,* the results showing the enhanced susceptibility of *noxy76* to pathogen infection suggest the participation of the ILA protein in regulating protein translation during pathogen infection. In this context it is possible to speculate that a high amino acid demand will take place during the activation of plant immunity needed to synthesize high amounts of defense proteins and by regulating translation of specific transcription factors. In this manner, the GCN1 protein will contribute to balance the amino acid requirements of the cell to cope with pathogen infection while maintaining cell viability. In addition, it is also possible that GCN1 could be involved in controlling the translation of additional transcription factors helping to accommodate the proper activation of plant defense.

5.9. *noxy76* is affected in signaling responses to oxylipins and singlet oxygen

Further characterization of the *noxy76* mutant to investigate whether the defect to activate plants defense could be correlated with the lack of response to specific cellular signals demonstrated the diminished capacity of *noxy76* to respond to the application of 9-HOT and 9-KOT as well as to singlet oxygen. As expected, the failure of *noxy76* to signal 9-LOX responses was evident from the results that showed that *noxy76* accumulated lower levels of 9-HOT and 9-KOT responding transcripts (*POX*, a FAD-dependent pyridine nucleotide-disulphide oxidoreductase;

FOX, a FAD-binding Berberine family protein with oxidoreductase activity and *TOUCH3,* a Calmodulin-like protein) than the wild type Col-0 plants (Figure 21).

In a similar manner and as previously found in *lox1 lox5* plants, the *noxy76* mutants showed a diminish response to singlet oxygen (Figure 23). Thus the *noxy76* showed enhanced cellular damage to this reactive oxygen species and reduced activation of gene expression. The partial insensitivity of *noxy76* to singlet oxygen was in accordance with the role of the 9-LOX pathway in controlling oxidative stress and supports the importance of this pathway in controlling lipid peroxidation (López *et al*., 2011), a critical event that needs to be tightly regulated in order to maintain the proper equilibrium between the production of ROS and the activation of the signaling mechanisms helping to control the damage produced by these reactive compounds and to activate plant defense (Mittler *et al*., 2004; Van Breusegem and Dat, 2006). In this context it can be speculated that the yellow leaf phenotype associated with the mutation in *NOXY76* could be attributed to uncontrolled lipid peroxidation.

5.10. *noxy76* is impaired in production and signaling nitric oxide

Since nitric oxide has a bi-functional role during plant pathogen interactions as an activator of stomatal closure and inducer of defense gene expression, it was of interest to examine whether the *noxy76* mutant could have a defect in the production of NO or in the signaling pathway regulated by this reactive compound.

Studies on the response of *noxy76* to the NO generator, SNP, revealed that this compound induced root growth arrest in both, wild type and *noxy76*, but that response was stronger in *noxy76* seedlings (Figure 24A and B). These results could be indicative of an enhanced response of *noxy76* to NO or otherwise that this mutant was partially insensitive to NO as compared with wild type plants. The fact that the induction of NO-responding transcripts examined here (*ABC, FOX, POX, LOX1* and *MDC*) was significantly lower in *noxy76* than in wild type plants, indicated that *noxy76* was impaired in NO signaling and that the root growth arrest phenotype observed was due to the failure of this mutant in signaling the response to this reactive compound (Figure 24C).

The failure of *noxy76* in responding to NO could explain the defects of this mutant in inducing both apoplastic and pre-invasive defense. In addition, further characterization of *noxy76* revealed a clear defect in the production of NO. Thus, we found a strong production of NO in the stomata of wild type plants responding to the virulent and coronatine deficient strains of

Pseudomonas, whereas no staining was observed in the case of *noxy76* (Figure 25). These results indicated the failure of *noxy76* in producing NO and suggest the increased susceptibility of *noxy76* to *Pseudomonas syringae* is probably due to the impairment in both production and signaling of NO which ultimately permit increased bacterial entry and growth in the apoplast.

Taken together, the results of these studies revealed the enhanced susceptibility of *noxy76* to distinct bacterial strains of *Pseudomonas* and that the defect of this mutation affects the activation of pre-invasive and apoplastic defenses. Of interest the characterization of *noxy76* revealed its insensitivity to 9-LOX oxylipins and NO, two defense components found to play a role at the two layers of defense examined. Whether these two types of signaling molecules act through a common signaling pathway is presently unknown and it will further investigated in our laboratory. Independently of this, the identification of NOXY76 as a regulator of protein translation support the participation of this regulatory mechanism as a critical component of plant defense in which the proper balance of the plant resources is essential for survival.

Conclusions

6. CONCLUSIONS

1) The mutants *noxy3*, *noxy72* and *noxy76* are insensitive to the 9-LOX oxylipin, 9-hydroxy octadecatrienoic acid (9-HOT) as they did not exhibit the root waving phenotype and are impaired in the production of callose in response to 9-HOT.
2) *noxy3*, *noxy72* and *noxy76* were also partially insensitive to isoxaben and failed to induce lignification in response to this herbicide.
3) As compared with wild type plants, the *noxy3* mutant exhibited enhanced pre-invasive immunity upon surface inoculation with the coronatine deficient strain *Pst* DC3000 *COR*$^{-}$ AK87.
4) The gene *NOXY3* encodes LON1 protease which is localized in the mitochondria.
5) *noxy72* showed enhanced post-invasive immunity as the growth of the virulent strain of *Pseudomonas syringae* was significantly reduced as compared with wild type plants.
6) The gene *NOXY72* encodes a S-adenosyl methionine dependent methyl transferase, which is involved in the biosynthesis of ubiquinone.
7) *noxy76* was impaired in both, pre- and post-invasive immunity as it permitted increased growth of all the *Pseudomonas* strains examined, independent of the mode of inoculation employed. The gene *NOXY76* encodes a HEAT-repeat protein called ILITYHIA which is known to regulate protein translation in yeast.
8) *noxy76* was susceptible to the exogenous application of singlet oxygen and impaired in the signaling of responses to this reactive compound.
9) *noxy76* was susceptible to NO and it was impaired in signaling responses to NO.
10) The stomata of *noxy76* failed to produce nitric oxide in response to *Pseudomonas syringae* and remained unresponsive to the presence of bacteria and oxylipins, a defect that facilitated bacterial entrance and reduced plant defense.

Summary in Spanish

7. SUMMARY IN SPANISH

7.1. Resumen

Las oxilipinas constituyen una clase de metabolitos lipídicos que juegan un papel importante tanto en el desarrollo, como en la defensa de las plantas frente al ataque de microorganismos patógenos. La sintesis de oxilipinas se inicia mediante la oxidación de ácidos grasos a través de la acción de enzimas con actividad 9- y 13- lipoxigenasas y α-dioxigenasas, así como mediante oxigenación química. La ruta de síntesis de oxilipinas iniciada por la acción de las enzimas 9-LOX constituye un área reciente de investigación, en la que se ha podido demostrar su participación en la defensa frente a la infección de bacterias hemibiotrofas. En este estudio, hemos llevado a cabo un análisis genético con objeto de identificar los componentes de la ruta de señalización que se activa en respuesta a la producción de las oxilipinas sintetizadas por la acción de las 9-LOX, así como de identificar su participación en la respuesta de defensa vegetal. Mediante escrutinio genético de una población de *Arabidopsis thaliana* mutagenizada se identificaron tres mutantes, denominados *noxy3*, *noxy72* y *noxy76*, insensibles a la aplicación del derivado de las 9-LOX, ácido 9-hidroxioctadecatrienoico (9-HOT). La caracterización de la respuesta de estos mutantes al patógeno hemibiotrofo *Pseudomonas syringae* nos ha permitido determinar que los genes *NOXY3* y *NOXY72* actúan como reguladores negativos de la defensa vegetal, y que las mutaciones en dichos genes provocan el aumento de la resistencia de la planta en los estomas y en el apoplasto, respectivamente. Por otro lado, el mutante *noxy76* mostró un aumento de la susceptibilidad de la planta frente a la infección de *Pseudomonas*, que se manifestaba tanto a nivel de los estomas como del apoplasto. El mapeo de los mutantes *noxy3* y del *noxy72* permitió determinar que las mutaciones correspondientes afectaban a las proteínas mitocondriales LON1 y metil transferasa dependiente de S-adenosil metionina, respectivamente, mientras que la mutación *noxy76* se localizó en el gen *ILITYHIA* que codifica una proteína citoplásmica implicada en procesos de regulación post-transcripcional de la expresión génica. Una caracterización más detallada reveló que el mutante *noxy76* mostraba un defecto en el cierre estomático en respuesta a la infección bacteriana, que resultó ser independiente de la aplicación de ABA. Del mismo modo, la caracterización de las respuestas a la generación de ROS y NO reveló que la mutación *noxy76* afectaba a la señalización de la respuesta de la planta frente a la producción de oxígeno singlete y de NO, y que presentaba un claro defecto en la producción de NO en los estomas.

7.2. Introducción

7.2.1. Interacción planta-patógeno

A lo largo de la evolución las plantas han desarrollado mecanismos de defensa para evitar el ataque de los microorganismos patógenos que, en la actualidad, y dada su complejidad, se engloban dentro de lo que se denomina como sistema inmune vegetal. Este sistema de defensa incluye la presencia de barreras físicas (cutículas y pared secundaria) y químicas (compuestos antimicrobianos) preformadas, así como mecanismos activos en los que, a través de la activación de rutas específicas de señalización, se induce la producción de numerosos compuestos antimicrobianos, y se provoca la modificación y el reforzamiento de la pared celular, que van contribuir, en último término, a dificultar el desarrollo y la progresión del patógeno en la planta. (Dangl y Jones, 2001; Thomma y col., 2001; Hammond-Kosack y Parker, 2003).

7.2.2. Respuesta de defensa vegetal

La respuesta de inmunidad vegetal engloba a todos aquellos mecanismos de defensa que se activan en la planta en respuesta a la presencia de patógenos y se agrupan en dos categorías, denominadas resistencia basal y resistencia inducida.

El reconocimiento de epitopos patogénicos por receptores transmembrana específicos (*Pattern Recognition Receptors* o *PRR's*) es el primer y más importante aspecto de la defensa vegetal, ya que conduce a una respuesta inmune inespecífica que constituye la resistencia basal de las plantas. El mejor ejemplo de este tipo de inmunidad es el reconocimiento del péptido bacteriano flagelina por los dominios ricos en repeticiones de leucina de la proteína receptora vegetal Flagellin Sensing 2 (FLS2), un receptor transmembrana tipo kinasa (Gomez-Gomez y Boller, 2000) cuya activación conduce a la inducción de una gran cantidad de respuestas de defensa.

Durante la co-evolución húesped-patógeno, las plantas han desarrollado otro nivel de defensa basado en la detección de proteínas efectoras (Chisholm y col., 2006; Jones y Dangl, 2006). Cuando el microorganismo invasor es capaz de vencer la resistencia basal de la planta, ésta inicia una respuesta de defensa secundaria y más eficiente para evadir la virulencia del patógeno. Esta resistencia secundaria, conocida como *effector triggered immunity* o inmunidad inducida, se lleva a cabo en su mayor parte en el interior de la célula y consiste en la activación de genes de resistencia R (Jones y Dangl, 2006; Martin y col., 2003; Nimchuk y col., 2003). Las proteínas codificadas por estos genes son polimórficas y la mayoría de ellas se caracterizan por poseer un sitio de unión a

nucleótidos (NB) y dominios ricos en repeticiones de leucina (LRR domains) (Dangl y Jones, 2001). Estas proteínas son capaces de reconocer una gran variedad de efectores patogénicos y de activar mecanismos de resistencia en la planta.

Las respuestas immunes provocadas por las defensas basal e inducida son muy similares (Navarro y col., 2004; Tsuda y col., 2009; Hammond-Kosack y Parker, 2003; Jones y Dangl, 2006) pero, aun así, los componentes constitutivos de cada una de ellas y las rutas de señalización que participan en estas dos barreras inmunes pueden diferir (Zipfel y col., 2008; Thilmony y col., 2006; Truman y col., 2006; Navarro y col., 2004). En conjunto, ambas respuestas suponen flujos de iones a través de la membrana plasmática, la generación de intermediarios de especies reactivas de oxígeno, oxido nítrico, deposición de calosa, activación de kinasas y la transcripción de numerosos genes de defensa (Nurnberger y col., 2004; Apel and Hirt, 2004; Zhang y col., 2003; Luna y col., 2010; Droillard y col., 2004).

7.2.3. Oxilipinas

La generación de compuestos lipídicos, a partir de los ácidos grasos de la membrana celular, ha sido descrita tanto en células animales como vegetales, en las que dichos compuestos juegan un importante papel en la activación de la respuesta inmune. Además, los ácidos grasos y sus derivados constituyen, no sólo una importante fuente de energía, sino que su actuación como señales celulares contribuye a la regulación de procesos relacionados con el desarrollo de las plantas, así como en la adaptación de éstas a distintas condiciones de estrés (Feussner y Wasternack, 2002; Shah, 2005; Howe y Jander, 2008).

7.2.3.1. Síntesis de oxilipinas

Dentro de los derivados lipídicos caracterizados, las oxilipinas constituyen una familia de metabolitos secundarios, con estructuras y actividades biológicas diversas, que se originan mediante la oxidación de ácidos grasos, predominantemente los ácidos linoleico (18:2) y linolénico (18:3).

La oxidación de ácidos grasos ocurre, preferentemente, de forma enzimática mediante la acción de los enzimas lipoxigenasa y α-dioxigenasa (Hamberg y col., 1999; Feussner y Wasternack, 2002). Además, la incorporación de oxígeno en los ácidos grasos puede ocurrir mediante oxidación química, como consecuencia de su interacción con especies reactivas de oxígeno, tales como los radicales hidroxilos y el singlete de oxígeno. La oxidación no enzimática de ácidos grasos da lugar a

la producción de un grupo específico de oxilipinas, entre las que los denominados fitoprostanos han sido los más caracterizados (Mueller y Berger, 2009).

La oxidación enzimática de ácidos grasos da lugar a la formación de hidroperóxidos que constituyen, por tanto, los productos primarios de las reacciones catalizadas por las α-DOX y las lipoxygenasas. Dentro de los enzimas con actividad lipoxigenasa se distinguen dos grupos de isoenzimas, denominadas 9-LOXs y 13-LOXs, dependiendo de que la incorporación de oxígeno ocurra en el carbono 9 o 13 del ácido graso. Los hidroperóxidos derivados de la acción de las lipoxigenasas son modificados posteriormente por la acción de actividades enzimáticas secundarias (Feussner y Wasternack, 2002).

Los estudios realizados con objeto de identificar los genes que codifican los enzimas LOXs en plantas de *Arabidopsis* nos han permitido identificar la presencia de 6 genes lipoxigenasa de los que los designados como *LOX1* y *LOX5* codifican enzimas con actividad 9-lipoxigenasa, mientras que las cuatro restantes (*LOX2*, *LOX3*, *LOX4* y *LOX6*) corresponden a enzimas con actividad 13-lipoxigenasa (Bannenberg y col, 2009).

7.2.3.2. Funciones de las oxilipinas

El importante avance en nuestro conocimiento sobre la naturaleza de las oxilipinas, su producción y las acciones que realizan ha permitido determinar, que la expresión de una gran parte de los genes involucrados en la síntesis de oxilipinas se induce en la planta en respuesta a la infección por distintos patógenos, dando lugar a la acumulación de los compuestos correspondientes (Ponce de León y col., 2002; Andersson y col., 2006).

De forma generalizada, la caracterización funcional de oxilipinas durante la defensa de la planta, ha permitido distinguir la capacidad de determinados derivados para ejercer tres tipos de actividades: una actividad señalizadora que conduce a la inducción de genes específicos de la planta, una actividad reguladora del proceso de muerte celular que acompaña a la inducción de la respuesta de defensa vegetal y, finalmente, una acción directa como moléculas con actividad antimicrobiana. (Vollenweider y col., 2000; Stintzi y col., 2001; Montillet y col., 2005; Prost y col., 2005; Andersson y col., 2006).

Según indicamos anteriormente, dentro de esta familia de metabolitos, los jasmonatos han sido extensamente caracterizados y su participación en distintas respuestas, tales como la defensa

frente a necrotrofos, la respuesta a herida mecánica, la protección frente a insectos y la maduración del polen, ha sido ampliamente demostrada (Staswick, 2008; Fonseca y col., 2009; Browse, 2009).

Además, existen datos recientes que demuestran la participación de las rutas iniciadas por la acción de las α-DOXs y 9-LOXs, así como de los productos sintetizados a través de dichas rutas en la defensa vegetal. El papel de la ruta 9-LOX en la defensa de tabaco y *Arabidopsis* se ha puesto de manifiesto mediante la utilización de plantas transgénicas, alteradas en los niveles de expresión de los genes correspondientes, y de plantas mutantes, carentes de dichas actividades enzimáticas (Andersson y col., 2006; Vellosillo y col., 2007; Hwang y Hwang, 2010; López y col., 2011).

Los resultados descritos ponen de manifiesto que, a pesar de los recientes avances en el estudio de las rutas de oxilipinas iniciadas por la acción de 9-LOX, así como de sus derivados, el conocimiento de estos compuestos y de la acción que realizan, es todavía limitado. El presente trabajo ha sido realizado con objeto de profundizar en el estudio de esta ruta de síntesis de oxilipinas así como de identificar los componentes celulares implicados en la señalización que se activa por la acción de los correspondientes derivados lipídicos y que forma parte de los mecanismos de defensa de la planta en respuesta a la infección de microorganismos patógenos.

7.3. RESULTADOS Y DISCUSIÓN

Con objeto de estudiar la ruta de señalización activada por la acción de las enzimas 9-LOX, procedimos a desarrollar una estrategia genética dirigida a aislar plantas mutantes, afectadas en dicha ruta de señalización, empleando para ello una población de plantas de *Arabidopsis thaliana* mutagenizadas con EMS, y utilizando como criterio de selección la insensibilidad a la aplicación del derivado de las 9-LOX, el ácido 9-hidroxioctadecatrienoico (9-HOT). Las plantas mutantes seleccionadas se denominaron *noxy*, de *non responding to oxylipin* y tres de ellas *noxy3*, *noxy72* y *noxy76* fueron seleccionadas para su caracterización en este trabajo.

La insensibilidad de los mutantes *noxy3*, *noxy72* y *noxy76* a la aplicación de 9-HOT estaba acompañada de un defecto en la acumulación de calosa. Por otro lado, la caracterización de dichos mutantes reveló su insensibilidad parcial a la aplicación de isoxaben y una disminución en la producción de lignina en respuesta a la aplicación de dicho herbicida. La producción de ambos polímeros, callosa y lignina está asociada a la activación de una respuesta defensa frente a la infección de patógenos (Brown y col., 1998; Schreiber y col., 1999), por lo que el defecto en la producción de calosa y lignina permitía suponer que las mutaciones examinadas podrían provocar una alteración de dicha respuesta y, posiblemente, el aumento de la susceptibilidad de las plantas frente a la infección de patógenos.

El análisis de los mutantes *noxy3*, *noxy72* y *noxy76* permitió determinar que las mutaciones examinadas modificaban la respuesta de la planta frente a la infección de distintas cepas de *Pseudomonas syringae*, y que dichos cambios se manifestaban en las dos barreras de defensa, estomática y apoplastica, que se activan en respuesta a la infección de este tipo de patógenos. Por otro lado, los resultados de estos análisis permitieron determinar que los cambios observados no siempre conducían al aumento de la susceptibilidad sino que, por el contrario, las mutaciones *noxy3* y *noxy72* provocaban un aumento en la capacidad defensiva de la planta. Así, mientras que el mutante *noxy3* se comportaba igual que las plantas silvestres en respuesta a la infiltración en el apoplasto de la planta de cepas virulentas y avirulentas de *Pst* DC3000, dicho mutante mostraba un aumento en la defensa que se activa en los estomas de la planta para evitar la entrada de patógenos bacterianos. La caracterización del mutante *noxy72* reveló un aumento de la respuesta de defensa que se activa en el apoplasto de la planta que permitía reducir el crecimiento de la bacteria *Pst* DC3000 en comparación al detectado en las plantas control, Esta resistencia estaba acompañada de una reducción en los niveles de acumulación de H_2O_2 que reflejaba una alteración de los mecanismos celulares que controlan la producción y/o señalización de ROS.

El clonaje posicional de los mutantes *noxy3* y *noxy72* puso de manifiesto la localización de las mutaciones correspondientes en los loci At5g26860 y At5g57300, que codifican las proteínas mitocondriales, LON1 y metiltransferasa dependiente de S-adenosil-L-metionina, respectivamente. La proteasa LON1 está implicada en la regulación de la homeostasis redox celular en mamíferos, donde actúa degradando proteínas modificadas en respuesta al estrés oxidativo que se produce durante las respuestas de hipoxia o de estrés oxidativo en el retículo endoplasmático (Ondrovicova y col., 2005). La proteína NOXY72 está implicada en la síntesis de ubiquinona, proteína que juega un papel central en el transporte de electrones en la mitocondria, en donde se reduce a ubiquinol por los complejos I y II (Popov y col., 2001). El ubiquinol actúa como quelante de ión superóxido por lo que la proteína NOXY72 podría estar involucrada en el control de las respuestas a estrés oxidativo durante la señalización de 9-HOT y el transporte electrónico mitocondrial.

La función de las proteínas NOXY3 y NOXY72 estaría en relación con los resultados previos que muestran el papel de la ruta de las 9-LOX en el control de la homeostasis de ROS. El aumento de la resistencia de estos mutantes podría ser consecuencia de una alteración en la producción de ROS y en los mecanismos de señalización que dirigen las respuestas a estos compuestos reactivos. Está idea se ve apoyada por el hecho de que las proteínas NOXY3 y NOXY72 se localizan en la mitocondrias cuya función en la producción de ROS y en su regulación ha sido ampliamente demostrada. En este contexto cabe mencionar que además de los mutantes *noxy3* y *noxy72*, los trabajos realizados en el laboratorio han permitido determinar que otros cuatro mutantes *noxy* corresponden a mutaciones en genes que codifican proteínas mitocondriales. Estos resultados, junto a los obtenidos en ensayos adicionales en los que se demuestra que el 9-HOT provoca la despolarización de la membrana mitocondrial, ponen de manifiesto la importancia de estos orgánulos en los procesos de señalización activados por esta oxilipina. Por otro lado, estos resultados contribuyen a sustentar los trabajos que ponen de manifiesto la importancia de la mitocondria y de las funciones mitocondriales en la activación de la inmunidad vegetal (Gleason y col., 2011; Schwarzlander y col., 2012; Arnoult y col., 2011). Por tanto, en línea con la discusión, el hecho de que los mutantes *noxy* mostraran una alteración en su capacidad para superar una infección patogénica, apoya el papel de la mitocondria en esta respuesta e indica que los mutantes *noxy* pueden considerarse como una herramienta importante para lograr un mayor conocimiento del papel de estos orgánulos en la defensa vegetal, así como de las rutas de señalización que controlan la comunicación núcleo-mitocondria.

La caracterización del mutante *noxy76* puso de manifiesto el aumento en el nivel de susceptibilidad frente a la infección bacteriana asociada a esta mutación. Dicho efecto afectaba de

la misma manera a todas las cepas bacterianas examinadas y era independiente del modo de infección utilizado. Así, los resultados derivados de nuestro trabajo ponían de manifiesto la participación de la proteína NOXY76 como parte de los mecanismos de defensa que se activan en los espacios intercelulares de la planta, para controlar el crecimiento bacteriano, y en los estomas para prevenir la entrada de patógenos y demostraban el papel de la proteína NOXY76 como un regulador positivo de la resistencia vegetal.

Durante la respuesta a la infección de la bacteria en el apoplasto de la planta el mutante *noxy76* mostraba una alteración en la acumulación de peróxido de hidrógeno similar a la caracterizada en los mutantes *lox1 lox5* y *noxy22*, en los que el aumento de la susceptibilidad a *Pst* DC3000 estaba acompañado del aumento en la acumulación de peróxido de hidrógeno y de un defecto en la activación del proceso de muerte celular que acompaña a la respuesta de defensa de la planta (López y col., 2011). Por otro lado, en el caso de la inoculación por pulverización el mutante *noxy76* presentaba un defecto en el cierre de los estomas que mantenían un nivel de apertura similar al de las plantas no infectadas.

Estudios recientes no publicados, han puesto de manifiesto la participación de los enzimas 9-LOX como parte de los mecanismos de la planta que determinan el cierre de los estomas en respuesta a una infección bacteriana. Esta función se basa en resultados que demuestran que: i) el gen *LOX1* se expresa en las células guarda de los estomas, ii) la ausencia de actividad 9-LOX está asociada a un defecto en el cierre de los estomas y iii) que oxilipinas, tales como el 9-KODE y 9-KOTE generadas a través de la ruta 9-LOX, eran capaces de inducir el cierre de los estomas a concentraciones nanomolares. En base a estos resultados pudimos comprobar que el mutante *noxy76* era incapaz de cerrar los estomas en respuesta a la aplicación de 9-KOT y 9-KOD (Figura 20) y que presentaba, por tanto, un defecto en la respuesta de cierre estomático que se activa en respuesta a estas oxilipinas. Estos resultados apoyan el papel de la ruta de las 9-LOX en la defensa pre-invasiva, así como la participación de la proteína NOXY76 en esta respuesta.

El clonaje posicional del mutante *noxy76* permitió identificar una mutación en el locus At1g64790, correspondiente al gen *ILITYHIA* (*ILA*), identificado previamente en una búsqueda de genes involucrados en el desarrollo del gametofito femenino y del saco embrionario. El análisis de la secuencia de la proteína ILA reveló un nivel significativo de homología con GCN1 (General Control Nonderepressible1), un regulador de la traducción proteica que controla la respuesta al ayuno de aminoácidos. La caracterización de esta respuesta en levaduras y humanos ha demostrado la capacidad de GCN1 de percibir e interaccionar con tARNs no cargados que se

acumulan durante la privación de amino ácidos para iniciar una cascada de señalización que induce, en último término, la expresión de genes de biosíntesis de aminoácidos (Sattlegger y Hinnebusch 2005).

Una caracterización exhaustiva del mutante *noxy76*, a fin de investigar si el defecto en la activación de la defensa vegetal podía estar relacionado con la falta de respuesta a señales celulares específicas, demostró la insensibilidad de este mutante a la aplicación de 9-HOT y 9-KOT, así como al singlete de oxígeno. El mutante *noxy76* acumulaba niveles menores que las plantas silvestres de los transcritos correspondientes a los genes de respuesta a 9-HOT y 9-KOT *POX* (que codifica una piridina nucleótido-disulfito oxidorreductasa dependiente de FAD), *FOX* (que codifica una proteína de la familia de las Berberine de unión a FAD con actividad oxidoreductasa) y *TOUCH3* (que codifica una proteína tipo Calmodulina). Además, mostraba un aumento del daño celular provocado por el singlete de oxígeno y una reducción de la activación de la expresión génica en respuesta al tratamiento con este tipo de especies reactivas de oxígeno. La insensibilidad parcial del mutante *noxy76* al singlete de oxígeno concuerda con el papel de la ruta de las 9-LOX en el control del estrés oxidativo y apoya la importancia de esta ruta de síntesis de oxilipinas en el control de la peroxidación lipídica, un proceso cuya regulación permite mantener el equilibrio necesario entre la producción de ROS y su utilización como señales celulares durante la activación de la respuesta de defensa de la planta.

Dado el papel bifuncional del óxido nítrico (NO) en los procesos de inmunidad vegetal en los que contribuye de forma positiva en el cierre de los estomas y en la inducción de la expresión de genes de defensa, se consideró interesante examinar si el mutante *noxy76* pudiera tener un defecto en la producción de NO o en la ruta de señalización regulada por este compuesto reactivo (Neill y col., 2002; Beligini y Lamattina, 2001). Los resultados derivados de estos estudios revelaron el aumento de la susceptibilidad del mutante *noxy76* a la aplicación de NO que se manifestaba por un aumento en la inhibición del crecimiento de la raíz en comparación al observado en las plantas silvestres. Además, el análisis de la expresión de genes de respuesta a NO puso de manifiesto que el mutante *noxy76* acumulaba niveles de transcritos inferiores a los detectados en las plantas silvestres y por tanto que el mutante *noxy76* presentaba un defecto en la ruta de transducción activada en respuesta a este compuesto. La incapacidad del *noxy76* para responder a NO explicaría el defecto en la inducción de las defensas pre-invasiva y apoplástica de este mutante. Finalmente, los resultados de nuestro trabajo han puesto de manifiesto el aumento de la producción de NO en los estomas de plantas silvestres en respuesta a la presencia de las cepas bacterianas virulenta y deficiente en coronatina de *Pseudomonas* examinadas en nuestros estudios y que dicha producción

no se observaba en el mutante *noxy76*. Estos resultados señalan el defecto del *noxy76* en la producción de NO y sugieren que el aumento en la susceptibilidad de este mutante a *Pseudomonas syringae* se debe, al menos en parte, al fallo en la producción y en la señalización de NO de esta planta que, en último término, facilita la entrada a través de los estomas y el crecimiento de las bacterias en el apoplasto.

7.4. CONCLUSIONES

1) Los mutantes *noxy3*, *noxy72* y *noxy76* son insensibles a la aplicación de 9-hidroxioctadecatrienoico (9-HOT), un derivado lipídico generado a través de la activación de la ruta de síntesis de oxilipinas iniciada por la acción de los enzimas 9-lipoxigenasas.
2) Los mutantes *noxy3*, *noxy72* y *noxy76* son parcialmente insensibles a isoxaben y presentan un defecto en la producción de lignina en respuesta a la aplicación de este herbicida.
3) En comparación con las plantas silvestres, el mutante *noxy3* presenta un aumento en la resistencia pre-invasiva frente a la infección de la cepa *Pst* DC3000 *COR*$^{-}$AK87 deficiente en la producción de coronatina.
4) El gen *NOXY3* codifica una proteasa LON1 de localización mitocondrial.
5) El mutante *noxy72* presenta un aumento en la resistencia de la planta a nivel del apoplasto, que se manifiesta mediante la reducción del crecimiento de la cepa virulenta *Pst* DC3000 en relación al nivel crecimiento alcanzado en las plantas silvestres.
6) El gen *NOXY72* codifica una metil tranferasa dependiente de S-adenosil metionina involucrada en la biosíntesis de ubiquinona.
7) El mutante *noxy76* manifiesta un aumento de la susceptibilidad de la planta tanto a nivel estomático, como apoplástico, que se manifiesta por el aumento del crecimiento de las cepas de *Pseudomonas* analizadas en este trabajo. El gen *NOXY76* codifica una proteína con repeticiones tipo HEAT denominada ILITYHIA que participa en la regulación de la traducción proteica.
8) El mutante *noxy76* es susceptible a la aplicación exógena de singlete de oxígeno y está afectado en la señalización de la respuesta de la planta frente a este compuesto reactivo.
9) El mutante *noxy76* es susceptible a NO y está afectado en las respuestas de señalización controladas por este compuesto.
10) Los estomas del mutante *noxy76* presenta un defecto en la producción de NO que impide su cierre en respuesta a la presencia de bacterias y de oxilipinas, y que facilita la penetración de patógenos y la reducción de la defensa vegetal.

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Appendix

Emerging Complexity in Reactive Oxygen Species Production and Signaling during the Response of Plants to Pathogens[1]

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Plants have evolved a complex immune system to perceive microbial pathogens and respond by producing defense compounds preventing infection. Defense hormones such as salicylic acid (SA), jasmonates, and ethylene are key signals regulating the production of antimicrobial defenses. Moreover, other hormone pathways have critical actions by controlling responses to pathogen attack such as distribution of resources, cell death, water stress, or plant architecture. A fine-tuning regulation of these pathways through complex regulatory networks is necessary to achieve resistance against different pathogen classes (López et al., 2008; Grant and Jones, 2009).

Plants activate two forms of immunity by recognition of distinct pathogen molecules. A first and rapid response, known as basal resistance (microbe-associated molecular pattern-triggered immunity or MTI), is triggered after recognition of conserved microbial molecules (microbe-associated molecular patterns) by extracellular plant receptors (pattern recognition receptors; Boller and Felix, 2009). Second, effector-triggered immunity (ETI) is activated by resistance (*R*)-gene products (largely inside the cell) after recognition of specific effectors molecules (delivered into the plant cell by pathogens) and is commonly accompanied by a hypersensitive reaction (HR) involving localized host cell death at the point of infection (Jones and Dangl, 2006).

Oxidative burst involving production of reactive oxygen species (ROS) is a nearly ubiquitous response of plants to pathogen attack and has a key role in both MTI and ETI signaling (Torres et al., 2006). ROS activation is likely a primary consequence of the damage produced during the course of infection. However, whereas overaccumulation of ROS might enhance plant susceptibility (Govrin and Levine, 2000; Kariola et al., 2005) or cause an uncontrolled defense with spreading cell death lesions that can kill the plant (Lorrain et al., 2003; Moeder and Yoshioka, 2008), a tight regulation over ROS production and elimination through enzymatic and nonenzymatic antioxidants has allowed plants to use these reactive compounds as a critical feature of MTI and ETI (Torres et al., 2006). Reported defense responses associated with the production of ROS include direct killing of pathogens, activation of host cell death (HR), and contribution to cell wall strengthening (Bolwell and Daudi, 2009). Moreover, emerging data highlight the role of ROS as signals in MTI and ETI (Torres et al., 2006; Van Breusegem et al., 2008) as well as their contribution to provide an appropriate redox environment needed to activate defense (Tada et al., 2008). The importance of ROS in plant defense will be discussed here with a focus on the production of distinct types of ROS and on the cellular compartments involved in their production.

ESTABLISHED ROLE FOR SUPEROXIDE IONS AND HYDROGEN PEROXIDE IN RESPONSE TO PATHOGEN ATTACK

Two distinct reactions can convert ground state oxygen into different types of ROS during an oxidative burst (Fig. 1). Thus, dioxygen can be stepwise reduced by electron transfer to superoxide ion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and the later can produce the hydroxyl radical ($OH^{\bullet}$). Alternatively, dioxygen can be converted by energy transfer to singlet oxygen ($^1O_2$), a highly reactive short-lived product (half-life, approximately 200 ns) with a strong oxidizing potential (Apel and Hirt, 2004). Production of ROS occurs at different cellular locations in response to distinct environmental cues, and both the scavenging mechanisms and signaling events triggered by $O_2^{\cdot-}$, H_2O_2, and $^1O_2$ have been investigated (op den Camp et al., 2003; Mittler et al., 2004; Gadjev et al., 2006). Results from transcriptomic analyses define common and specific responses toward different types of ROS as well as cross talk between distinct ROS signaling pathways, pointing to a complex scenario in which a fine regulation is critical for plant survival (op den Camp et al., 2003; Gadjev et al., 2006; Laloi et al., 2007).

Research examining the role of ROS in plant defense has been focused on the actions of $O_2^{\cdot-}$ and H_2O_2, whereas other ROS such as $OH^{\bullet}$ and $^1O_2$ have been far less examined (Apel and Hirt, 2004). Different

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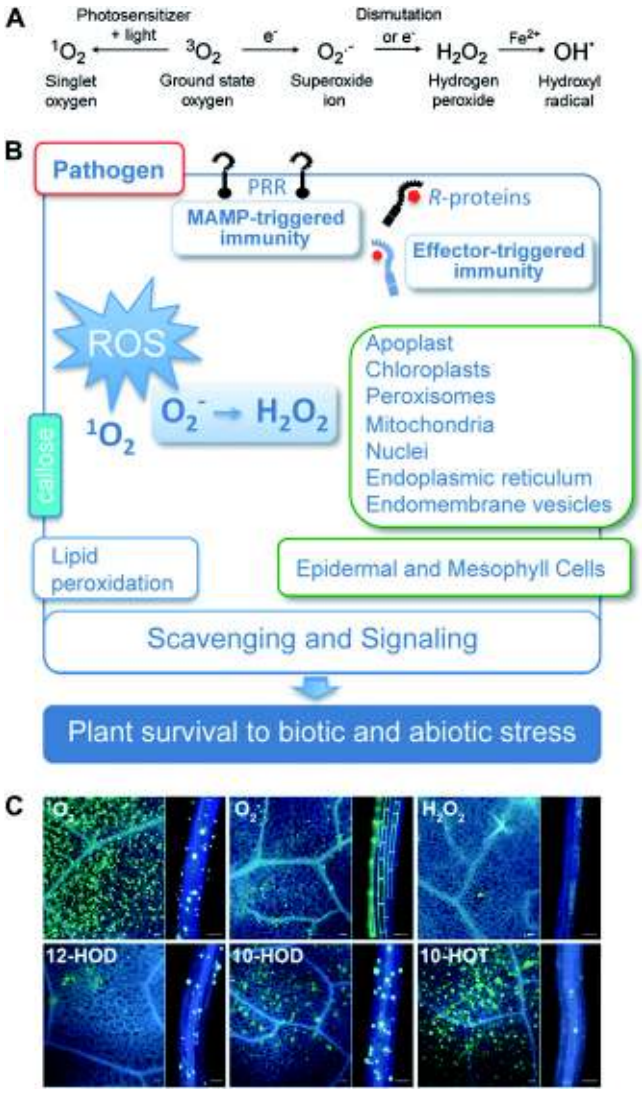

Figure 1. Production of ROS in plant defense. A, Generation of distinct ROS during oxidative burst. B, Tight regulation of ROS production at different cellular compartments and cell types is critical for plant survival. C, Callose accumulation after production of $^1O_2$, O_2^-, H_2O_2, and specific $^1O_2$-derived hydroxy fatty acids. Aniline blue staining in Arabidopsis leaves of 4-week-old plants (square-shaped sections) and in roots of in vitro-grown seedlings (rectangular sections). Leaves were infiltrated with Rose Bengal (1 μM as $^1O_2$ producer), xanthine-xanthine oxidase (2 mM-0.1 units per mL as extracellular O_2^- generator), H_2O_2 (1 mM), 12-HOD (25 μM), 10-HOD (25 μM), and 10-HOT (25 μM). Roots are from 8-d-old seedlings grown in Murashige and Skoog medium and covered with a solution of the ROS inducers xanthine-xanthine oxidase and H_2O_2, at the above concentrations, or germinated 4 d in Murashige and Skoog medium and then transferred to a fresh medium containing Rose Bengal (100 nM), or 12-HOD, 10-HOD, and 10-HOT (10 μM). Representative examples of 24 h treated tissues are shown in all cases. Scale bars = 50 μm.

enzymes have been implicated in the generation of apoplastic ROS in plant defense, among which NADPH oxidases (also known as respiratory burst oxidases or Rbohs), similar to those present in mammalian neutrophils, have received most attention. Plant NADPH oxidases catalyze the formation of superoxide by the following reaction: NADPH + $2O_2$ = $NADP^+ + H^+ + 2O_2^-$. Secondary spontaneous or superoxide dismutase-catalyzed conversion of superoxide provides H_2O_2, which in turn can afford $OH^\bullet$ in the presence of transition metal ions such as Fe^{2+} or Cu^+. Genetic analysis demonstrated that reduction or lack of RbohD and RbohF leads to elimination of extracellular H_2O_2 (Torres et al., 2002). However, reduced production of O_2^- and of its dismutation product H_2O_2 exerts different effects in plant pathogen growth and HR cell death, which suggests that apoplastic ROS might interact with distinct signaling pathways to serve different purposes. Thus, the spreading lesion phenotype of the *lsd1* mutants (for *lesion stimulating disease*) is enhanced in the triple mutant *lsd1-rbohD-rbohF*, which has led to propose the role of RbohD and RbohF in limiting SA-elicited cell death in cells surrounding an infection site (Torres et al., 2005).

In addition to the apoplast, evidence for a role of chloroplast, peroxisomes, or mitochondria in ROS production has been reported (Van Breusegem et al., 2008). Moreover, recent studies identified other cellular sites such as endoplasmic reticulum, endomembranes vesicles, and nuclei as producers of ROS during pathogen responses, although the actions of ROS from these cellular locations remain mostly unknown (Ashtamker et al., 2007). The participation of chloroplasts in pathogen responses is concluded by results showing that light is required to activate defense gene expression and HR (Karpinski et al., 2003) and that the light-growth conditions might affect the formation of infection-like lesions in a number of Arabidopsis (*Arabidopsis thaliana*) mutants (Lorrain et al., 2003; Moeder and Yoshioka, 2008). In many cases, these phenotypes correlate with a failure in the photosynthetic machinery or in the mechanisms protecting cells against oxidative damage, including the process of photorespiration that mitigates photooxidative damage and requires the participation of peroxisomes and mitochondria (Moreno et al., 2005; Queval et al., 2007).

The role of Enhanced Disease Susceptibility1 (EDS1) as a master regulatory protein that coordinates defense by processing chloroplastic ROS-derived signals has been shown (Straus et al., 2010). However, chloroplastic ROS production and plant defense can be uncoupled. A recent example is the demonstration that chloroplast-derived ROS are essential for the formation of HR cell death but not for the activation of other basal defense responses in tobacco (*Nicotiana tabacum*) transgenic plants (Zurbriggen et al., 2009). Also, mutation of the chloroplastic Resistance to Phytopthora1 protein in Arabidopsis led to reduced H_2O_2 and enhanced susceptibility to *Phytopthora brassicae*, but caused a rapid run away cell death that originated at the point of infection (Belhaj et al., 2009).

Like chloroplasts, the mitochondria can also be an important source of ROS during physiological or pathological conditions that possess an efficient antioxidant machinery to control their toxic effects (Apel

and Hirt, 2004). Nevertheless, the role of these organelles in plant cell death and pathogen responses has received little attention. Several studies revealed that treatments with cell death inducers, such as bacterial elicitors or virulence effectors might disrupt the functionality of the mitochondria and increase basal levels of ROS (Balandin and Castresana, 2002; Yao et al., 2004; Block et al., 2010). These results suggest that mitochondrial disturbance is a broadly employed strategy by pathogens to suppress host immunity and that increased ROS may contribute to the protection of plants against pathogen damage.

Results described above indicate that plants have evolved sophisticated mechanisms to use the compartmentalized production of ROS in the modulation of the defense responses against pathogen attack.

EMERGING ROLES FOR $^1O_2$ AND LIPID PEROXIDATION IN PLANT DEFENSE

Information on the role of $^1O_2$ in plant defense is still very limited. However, direct and indirect evidence discussed below, is starting to disclose a signaling role of $^1O_2$ and its participation in the response to pathogens, an area that can be expected to receive much attention in the near future. $^1O_2$ is a highly reactive unstable molecule produced in plants under basal and light stress conditions (Triantaphylidès and Havaux, 2009). In the chloroplast, excited chlorophyll can act as a photosensitizer to produce $^1O_2$ from ground state oxygen. In addition, secondary metabolites such as phenaleno-like phytoalexins and phytoanticipins might act as photosensitizers to generate $^1O_2$ following absorption of light energy (Flors and Nonell, 2006). Increased levels of these metabolites after pathogen attack could thus contribute to generate $^1O_2$ as a product of the plant defense machinery.

$^1O_2$ has a crucial role during acclimation of plants to high light intensity and photooxidative stress (Triantaphylidès et al., 2008), a response that shows strong similarities to plant defense, including the functional integration of SA and of defense regulatory proteins such as LSD1, EDS1, and Phytoalexin Deficient4 (Mühlenbock et al., 2008). Of great interest, studies with the conditional *flu* mutant that generates $^1O_2$ upon light illumination (op den Camp et al., 2003) allow to distinguish two modes of $^1O_2$ activity. Thus, whereas high $^1O_2$ production leads to photooxidative damage, decreased levels mediate a signaling activity, two responses that could be executed by $^1O_2$ or by more stable $^1O_2$-dependent products (Przybyla et al., 2008).

A universal response of plants to pathogen attack is the generation of a host of active lipid derivatives, collectively known as oxylipins (Andreou et al., 2009; Mosblech et al., 2009). Such compounds can be formed either by enzymatic or nonenzymatic peroxidation of fatty acids, however, certain of the hydroxy oxylipins, i.e. linoleic acid-derived 10-hydroxy-octadecadienoic acid (10-HOD) and 12-HOD and linolenic acid-derived 10-hydroxy-octadecatrienoic acid (10-HOT) and 15-HOT, can only be formed by $^1O_2$-dependent nonenzymatic oxygenation and can therefore be used as in vivo markers of $^1O_2$ generation (Przybyla et al., 2008). Whereas oxylipins formed by both specific enzymatical pathways (Hamberg et al., 2005; Kachroo and Kachroo, 2009) and by nonenzymatical free-radical reactions (Loeffler et al., 2005) play important roles in plant defense, no function has yet been assigned to the $^1O_2$-derived hydroxy fatty acids. Of interest, these latter compounds accumulate in etiolated *flu* seedlings following illumination (Przybyla et al., 2008) and in leaves of Arabidopsis responding to *Pseudomonas syringae* pv *tomato* inoculation (Grun et al., 2007), reflecting the generation of $^1O_2$ during stress responses, photooxidation, and pathogen attack.

Deposition of callose is a frequent response of cells to pathogen assault (Hématy et al., 2009). Importantly, production of $^1O_2$ (triggered by Rose Bengal) and application of $^1O_2$-formed hydroxy acids, induce a strong accumulation of callose in leaves ($^1O_2$ and 12-HOT) and roots ($^1O_2$, 10-HOD, and 12-HOD) of Arabidopsis (Fig. 1). Callose deposition was also observed (preferentially in roots) after application of O_2^- (generated by xanthine-xanthine oxidase) and was only weakly detected in roots of seedlings responding to H_2O_2 (Fig. 1). These results suggest that ROS such as $^1O_2$ and O_2^- might contribute to the accumulation of callose during the response of plants to pathogen attack. Of interest, the differences in the pattern of callose deposition observed after generation of $^1O_2$ and O_2^- or application of distinct $^1O_2$-derived hydroxy fatty acids point to tissue-specific variations in the mode of action to these compounds. Further support of the participation of $^1O_2$ in plant defense comes from results showing an overrepresentation of biotic stress-related genes during the transcriptomic reprogramming activated after generation of $^1O_2$ (M. Martínez and C. Castresana, unpublished data).

Although these new observations deserve further investigation, these results are indicative of an active response of plants toward specific $^1O_2$-derived hydroxy fatty acids. Related to this, we note that the nonenzymatic oxidation of linolenic acid contributes to limit pathogen infection and spreading cell death and that the action of linolenic acid as a sink for ROS has been suggested (Mène-Saffrané et al., 2009). Also, in line with our discussion, we speculate that the $^1O_2$-derived hydroxy fatty acids could play a role in oxidative stress signaling and actively contribute to protect plant tissues against pathogen attack.

CONCLUDING REMARKS

Results described above reveal that plants have evolved unique defense responses that depend on ROS production and redox signals generated by different mechanisms at specific cellular locations. Tight control over production and accumulation of ROS is likely to be crucial to plants grown in natural envi-

ronments where survival to pathogen attack and acclimation to prevailing abiotic stress factors (like high light) have to be integrated. However, key aspect of ROS production and signaling remain still poorly understood. Plants generate chemically distinct oxygen derivatives, which may be selectively produced at specific cellular locations in response to different environmental stresses. Studies to investigate the actions of ROS have revealed common and specific responses toward different types of ROS as well as cross talk between distinct ROS signaling pathways, thus showing a complex scenario hampering the investigation of selective actions by a given ROS. Major efforts in examining the actions of O_2^- and H_2O_2 might have oversimplified the analyses of ROS in plant defense. Compared to other ROS, $^1O_2$ has received little attention, and recent studies indicating its participation in plant defense are emerging. Polyunsaturated fatty acids are a preferred target of $^1O_2$ attack and several of its oxidation products could act as secondary messengers to trigger defense responses. Studies on the actions of ROS will benefit from newly developed tools helping to monitor in a noninvasive manner, the generation and signaling events of distinct types of ROS in the activation of resistance after pathogen attack.

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Antagonistic role of 9-lipoxygenase-derived oxylipins and ethylene in the control of oxidative stress, lipid peroxidation and plant defence

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SUMMARY

9-lipoxygenases (9-LOXs) initiate fatty acid oxygenation in plant tissues, with formation of 9-hydroxy-10,12,15-octadecatrienoic acid (9-HOT) from linolenic acid. A *lox1 lox5* mutant, which is deficient in 9-LOX activity, and two mutants *noxy6* and *noxy22* (*non-responding to oxylipins*), which are insensitive to 9-HOT, have been used to investigate 9-HOT signalling. Map-based cloning indicated that the *noxy6* and *noxy22* mutations are located at the *CTR1* (CONSTITUTIVE ETHYLENE RESPONSE1) and *ETO1* (ETHYLENE-OVERPRODUCER1) loci, respectively. In agreement, the *noxy6* and *noxy22* mutants, renamed as *ctr1-15* and *eto1-14*, respectively, showed enhanced ethylene (ET) production. The correlation between increased ET production and reduced 9-HOT sensitivity indicated by these results was supported by experiments in which exogenously added ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) impaired the responses to 9-HOT. Moreover, a reciprocal interaction between ET and 9-HOT signalling was indicated by results showing that the effect of ACC was reduced in the presence of 9-HOT. We found that the 9-LOX and ET pathways regulate the response to the lipid peroxidation-inducer singlet oxygen. Thus, the massive transcriptional changes seen in wild-type plants in response to singlet oxygen were greatly affected in the *lox1 lox5* and *eto1-14* mutants. Furthermore, these mutants displayed enhanced susceptibility to both singlet oxygen and *Pseudomonas syringae* pv. *tomato*, in the latter case leading to increased accumulation of the lipid peroxidation product malondialdehyde. These findings demonstrate an antagonistic relationship between products of the 9-LOX and ET pathways, and suggest a role for the 9-LOX pathway in modulating oxidative stress, lipid peroxidation and plant defence.

Keywords: oxylipins, ethylene, oxidative stress, plant defence, Arabidopsis.

INTRODUCTION

Oxylipins are lipid-derived molecules that are ubiquitous in eukaryotes, and whose importance in controlling physiological and pathological processes in plants is being recognized (Savchenko *et al.*, 2010; López *et al.*, 2008). Enzymatic synthesis of oxylipins is initiated by incorporation of oxygen into a fatty acid molecule, catalysed by the activities of 9- and 13-lipoxygenases or α-dioxygenases (Andreou *et al.*, 2009; Hamberg *et al.*, 2005). The primary product is a fatty acid hydroperoxide, which is subsequently modified by an array of secondary enzymatic activities to generate an extensive family of metabolites (Mosblech *et al.*, 2009). Production of oxylipins from polyunsaturated fatty acids can also take place non-enzymatically in the presence of singlet oxygen or by free radical-mediated oxygenation (Durand *et al.*, 2009; Hamberg, 2011).

Synthesis of oxylipins is triggered at specific developmental stages, as well as by various biotic and abiotic stresses. During these responses, oxylipins were found to regulate gene expression (Stintzi *et al.*, 2001; Mueller *et al.*, 2008; Browse, 2009) and cell death (Vollenweider *et al.*, 2000; De León *et al.*, 2002; Hamberg *et al.*, 2003; Montillet *et al.*, 2005), and to exert direct anti-microbial activity (Prost

et al., 2005; Kishimoto *et al.*, 2008). Much attention has been paid to the biosynthetic pathway initiated by 13-lipoxygenases (13-LOX), and its main product, jasmonic acid (JA), which plays critical roles in fertility, resistance to necrotrophic pathogens, insect attack, wounding and establishing systemic resistance (Mandaokar and Browse, 2009; Caldelari *et al.*, 2011; Glazebrook, 2005; Onkokesung *et al.*, 2010; Koo and Howe, 2009; Truman *et al.*, 2007). A number of studies have also demonstrated the participation of oxylipins produced by the 9-LOX and α-DOX pathways in regulating plant development and defence responses (Rancé *et al.*, 1998; Vellosillo *et al.*, 2007; Gao *et al.*, 2008; Hwang and Hwang, 2010; De León *et al.*, 2002; Obregón *et al.*, 2001; Bannenberg *et al.*, 2009b). Furthermore, increasing evidence indicates a role for non-enzymatically generated oxylipins in plant defence (Loeffler *et al.*, 2005; Mueller and Berger, 2009). Nevertheless, the signalling mechanisms by which these oxylipins exert their function remain poorly understood.

Using a collection of 47 oxylipins and an *in vitro* assay, we identified three types of oxylipin-activated phenotypic alterations on root growth, i.e. root waving, loss of root apical dominance, and decreased root elongation. Among the oxylipins tested, the 9-LOX product 9(*S*)-hydroxy-10,12,15-octadecatrienoic acid (9-HOT) was the most potent inducer of root waving, and was used to identify 9-HOT-insensitive *noxy* mutants (*non-responding to oxylipins*) within a mutagenized Arabidopsis population. Studies using the 9-HOT-insensitive mutant *noxy2* and the JA-insensitive *coi1-1* mutant demonstrated that the response of plants to 9-HOT was activated by a JA-independent signalling pathway, and that the *noxy* mutants constitute a valuable tool to investigate the signalling components of the 9-HOT response (Vellosillo *et al.*, 2007).

Examination of the molecular events triggered by 9-HOT revealed accumulation of callose, production of reactive oxygen species (ROS), and transcriptional changes for genes involved in plant defence. These actions are common to the response to pathogen infection (Dangl and Jones, 2001), and are indicative of a role of 9-HOT in plant defence. In agreement with this, the 9-HOT-insensitive *noxy2* mutant showed enhanced susceptibility to *Pseudomonas syringae* pv. *tomato* (*Pst*). Moreover, *noxy2* mutants showed reduced necrosis after infection compared with wild-type plants. These findings were in line with previous results showing accumulation of 9-HOT, and additional 9-LOX derivatives, during activation of a hypersensitive cell-death response protecting plants against biotrophic pathogens, as well as in the cell-death reaction caused by pathogen elicitors (Vollenweider *et al.*, 2000; Hamberg *et al.*, 2003; Göbel *et al.*, 2003; Andersson *et al.*, 2006; Rustérucci *et al.*, 1999; Montillet *et al.*, 2005).

Here, we have characterized the signalling processes regulated by the 9-LOX derivative 9-HOT. We made use of a double mutant, *lox1 lox5*, that lack 9-LOX activity, and of two 9-HOT-insensitive mutants, *noxy6* and *noxy22* (*non-responding to oxylipins*), and uncovered an antagonistic role of 9-LOX and ethylene (ET) in controlling responses involving oxidative stress, lipid peroxidation and plant defence.

RESULTS

The *noxy6* and *noxy22* mutants, which are non-responsive to 9-HOT, are constitutive ethylene mutants

The 9-HOT-insensitive mutants *noxy6* and *noxy22* were isolated by a forward genetic screen based on the root waving activity of 9-HOT. We found that both mutants failed to respond to 9-HOT by inducing root waving but did respond to oxylipins such as JA and 9-oxononanoic acid (9-Oxo-C_9), which cause a reduction of root elongation (Figure S1). In addition, *noxy6* and *noxy22* plants did not activate responses to 9-HOT such as formation of focal deposits of callose (Figure S2). Moreover, production of superoxide anion and expression of three genes up-regulated by 9-HOT, i.e. *POX* (pyridine nucleotide oxidoreductase, At5g22140), *ABC* (ABC transporter, At1g15520) and *FOX* (FAD-binding oxidoreductase, At1g26380) (Vellosillo *et al.*, 2007), were reduced in these *noxy* mutants compared with wild-type plants (Figure S2).

Genetic analysis indicated that the *noxy6* and *noxy22* mutations were monogenic and recessive. Map-based cloning showed that the *noxy6* mutation is a C → T transition at nucleotide 3544 in the *CTR1* gene, which was renamed *ctr1-15* (Figure S3). The *ctr1-15* mutation converts Leu610 to Phe in the kinase domain of the CTR1 protein, which is a negative regulator of the ET pathway (Kieber *et al.*, 1993). The *noxy22* mutation is a C → T transition that converts Pro265 to Leu in the ETO1 protein, and was renamed *eto1-14* (Figure S3). The ETO1 protein is a negative regulator of ET synthesis (Wang *et al.*, 2004). As a consequence of the *ctr1-15* and *eto1-14* mutations, the ET pathway was constitutively activated, as confirmed by the characteristic triple response of dark-grown seedlings (Figure S4) (Guzmán and Ecker, 1990). These results indicate that ET may negatively interact with 9-HOT signalling.

Ethylene impairs the responses to 9-HOT via the canonical ethylene signalling pathway

Because constitutive ET production diminishes the response to 9-HOT, we examined the effect of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) on the 9-HOT response. We found that ACC impaired root waving, and reduced the accumulation of callose and production of superoxide anion that otherwise accompanied 9-HOT treatment (Figure 1). We also tested the response of the ET-insensitive mutant *ein2-5* (Alonso *et al.*, 1999) to application of 9-HOT, either alone or in combination with ACC. We found that *ein2-5* reacted as wild-type plants do to 9-HOT by

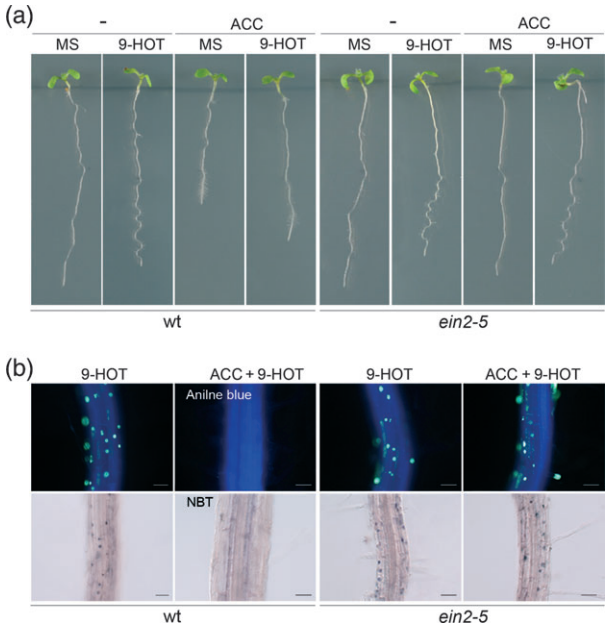

Figure 1. Characterization of wild-type and *ein2-5* plants in response to ACC and 9-HOT.
(a) Transmitted light visualization of seedlings grown for 3 days in MS medium and transferred to grow for an additional 3 days on MS medium containing 9-HOT (25 μM), ACC (2 μM) or 9-HOT in combination with ACC.
(b) Staining of roots treated as above. Fluorescence visualization of callose deposition in roots stained with aniline blue (upper panels), and transmitted light visualization of superoxide ions in roots stained with nitroblue tetrazolium (lower panels).

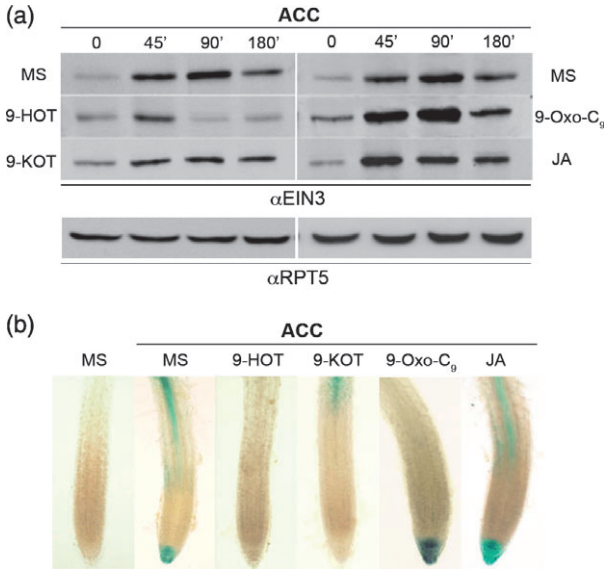

Figure 2. Analyses of ET signalling activation.
(a) Analyses of EIN3 protein accumulation in 6-day-old seedlings of *35S:EIN3-GFP* transgenic lines grown in MS medium and transferred to MS medium containing ACC (2 μM) alone or in combination with the oxylipins 9-HOT, 9-KOT, JA or 9-Oxo-C_9 (25 μM). Proteins were extracted and examined at the times indicated. Blots were hybridized to an antibody against EIN3. Anti-RPT5 was used as a loading control.
(b) Histological examination of GUS activity in transgenic lines containing the ethylene-responsive reporter *EBS:GUS*. GUS staining was performed 24 h after treatment with ACC alone or in combination with the oxylipins 9-HOT, 9-KOT, JA or 9-Oxo-C_9 (25 μM).

inducing root waving, callose deposition and ROS production. However, the effect of ACC on 9-HOT-activated root responses was impaired in the *ein2-5* mutant. These results indicated that activation of the 9-HOT responses examined does not require an active ET signalling pathway, but that the inhibitory effect of ACC on 9-HOT signalling is exerted through the canonical ET signalling pathway. Further, analysis of genes up-regulated by 9-HOT in *ein2-5* plants revealed enhanced accumulation of the three transcripts examined (*POX*, *ABC* and *FOX*) compared with wild-type plants (Figure S5). These results support a negative regulatory role of ET on the 9-HOT signalling pathway.

9-HOT interferes with activation of the ET signalling pathway

Because enhanced ET production inhibits 9-HOT signalling, it was of interest to examine whether, reciprocally, 9-HOT could antagonize ET signalling. In support of this idea, we noted that the inhibitory effect of ACC on root growth was diminished in the presence of 9-HOT (Figure 1a). To evaluate the effect of 9-HOT on the ET pathway, we made use of a well-characterized transgenic line *35S:EIN3-GFP* that shows constitutive expression of EIN3, a key transcription factor that is stabilized in response to ET to activate gene expression (Guo and Ecker, 2003). As shown in Figure 2(a), accumulation of EIN3–GFP increased strongly on application of ACC, and the increase was clearly reduced when 9-HOT was applied simultaneously with ACC. Further support for the effect of 9-HOT on the ET pathway was obtained using *EBS:*β-glucoronidase (GUS) transgenic plants, in which expression of the GUS gene is driven by a synthetic EIN3-responsive promoter (Stepanova *et al.*, 2007). As shown in Figure 2(b), no detectable expression of *EBS:GUS* was observed in roots of untreated plants, but ACC treatment resulted in a significant increase in GUS in root tips and in the elongation zone of the roots. The activation of *EBS:GUS* was reduced to a great extent when ACC was applied in combination with 9-HOT, proving that exogenous 9-HOT interferes with activation of the ET pathway.

In addition to 9-HOT, we explored the action of a second root waving oxylipin, i.e. 9-keto-10,12,15-octadecatrienoic acid (9-KOT), as well as of oxylipins that cause a reduction of root elongation, such as JA and 9-Oxo-C_9. We found that 9-KOT reduced the ACC-induced accumulation of EIN3, but to a smaller extent than 9-HOT, whereas JA and 9-Oxo-C_9 led to an increase in EIN3 compared with the level observed when ACC alone was applied (Figure 2a). Accordingly, activation of the *EBS:GUS* construct was significantly

reduced when ACC was applied in combination with 9-KOT, whereas no decrease of GUS activity was detected after treatment with JA or 9-Oxo-C_9 in combination with ACC (Figure 2b).

Lipid oxidation participates in the response of plants to 9-HOT

As 9-HOT increases ROS production and constitutive ET production (or application of ACC) prevents this response, we tested whether the cellular redox environment influences 9-HOT signalling. To this end, we compared the response of wild-type seedlings to 9-HOT when applied alone or in combination with various redox-active compounds, such as reduced glutathione (GSH), glutathione disulfide (GSSG; the oxidized form of glutathione), ascorbate (a key redox buffer) and Trolox (which has lipophilic anti-oxidant activity, Fluka, http://www.sigmaaldrich.com). None of the redox-active compounds analysed caused any visible phenotypic alteration compared with seedlings grown on MS (Figure S6). Moreover, we found that the application of ascorbic acid, GSH or GSSG (100 μM) did not cause major differences in the responses to 9-HOT, such as root waving, callose deposition and ROS production (Figure 3). In contrast, the three 9-HOT responses examined were inhibited when 9-HOT was applied in combination with Trolox (50 μM) (Figure 3). As with wild-type plants, Trolox inhibited the response to 9-HOT in *ein2-5* mutants, showing that the anti-oxidant activity of Trolox is exerted in an ET signalling-independent manner (Figure 3). The fact that application of Trolox, a tocopherol analogue with lipophilic anti-oxidant activity, inhibited the responses to 9-HOT suggests that oxidative stress and lipid peroxidation may be involved in 9-HOT signalling. On the other hand, the fact that tocopherols exert their lipid-protecting action by efficiently quenching singlet oxygen and scavenging various radicals (Triantaphylidès and Havaux, 2009) suggests the participation of 9-HOT in signalling responses to these reactive molecules.

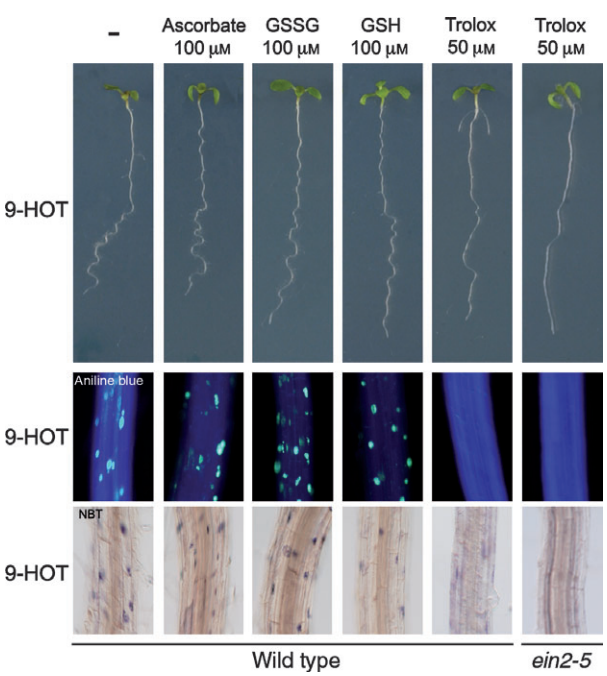

Figure 3. Effect of the redox environment on 9-HOT signalling.
(a) Phenotype of wild-type seedlings grown for 3 days in MS medium and transferred for an additional 3 days to MS medium containing redox-active compounds such as ascorbate, gluthatione disulfide (GSSG), reduced glutathione (GSH) and Trolox, in combination with 9-HOT (25 μM). Three-day-old seedlings of *ein2-5* grown in MS medium were transferred for an additional 3 days to MS medium containing Trolox (50 μM) in combination with 9-HOT (25 μM).
(b) Fluorescence visualization of callose deposition in roots stained with aniline blue (upper panels), and transmitted light visualization of superoxide anion in roots stained with nitroblue tetrazolium (lower panels).

Transcriptional changes in response to singlet oxygen varied significantly in 9-LOX and ET mutants compared to wild-type plants

Because of the involvement of lipid oxidation in 9-HOT signalling, we predicted that the response to singlet oxygen ($^1O_2$), a highly reactive oxygen species (ROS) that can initiate lipid peroxidation (Przybyla *et al.*, 2008; Triantaphylidès and Havaux, 2009), would differ between 9-HOT-deficient mutants and wild-type plants. Therefore, we examined the response of 9-LOX-deficient mutants and wild-type plants to Rose Bengal (RB), which is used to produce $^1O_2$. The *eto1-14* mutant (interfering with the 9-LOX pathway) was selected for these studies. In addition, a double mutant, *lox1-1 lox5-1* (hereafter referred to as *lox1 lox5*) containing a T-DNA insertion in each of the two 9-LOX genes of Arabidopsis, *LOX1* and *LOX5* (Bannenberg *et al.*, 2009a), was generated and included in these analyses (Figure S7). Quantification of 9-HOT to monitor 9-LOX activity revealed negligible amounts of 9-HOT in leaves and roots tissues of *lox1 lox5* seedlings. In addition, *eto1-14* seedlings showed levels of 9-HOT similar to those found in wild-type plants (Table 1).

The results from transcriptional profiles shown in Figure 4 (described in detail in Experimental Procedures) indicate that RB caused major transcriptional changes in gene expression, with 1262 genes being up-regulated and 570 genes down-regulated compared with mock-treated controls (twofold change or more false discovery rate 0.05) (Table S1). Comparison of transcriptional profiles with those obtained in previous studies using the conditional *flu*

Table 1 Levels of 9-HOT (nmol g^{-1}) in seedling homogenates incubated with linolenic acid

Genotype	Leaves	Roots
Wild-type	80.5 ± 11.5	133.2 ± 10.4
lox1 lox5	0.5 ± 0.04	0.6 ± 0.2
eto1-14	66.1 ± 1.4	102.8 ± 19.1

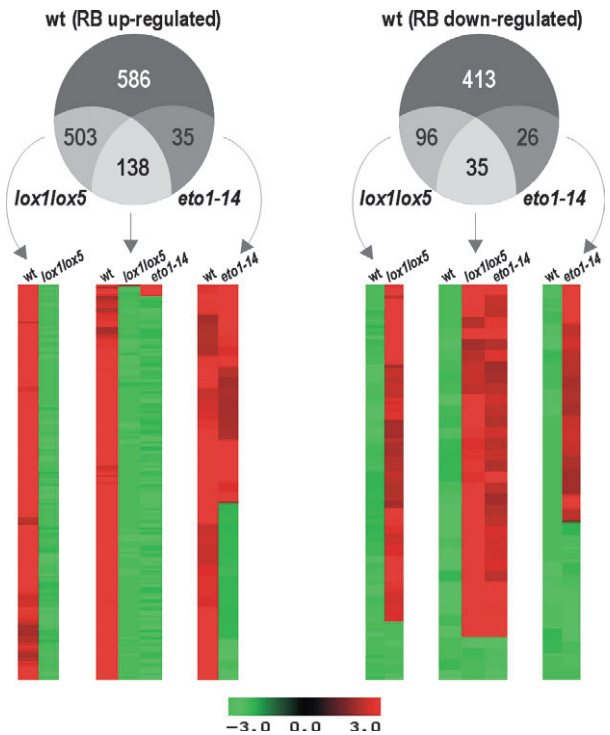

Figure 4. Microarray analyses of genes responsive to Rose Bengal (RB) in wild-type, *lox1 lox5* and *eto1-14* plants.
RNA samples were prepared from 12-day-old seedlings 3 h after Rose Bengal treatment (10 μM). Genes with twofold or greater differential expression that were either up-regulated or down-regulated in the plants examined were analysed. Venn diagrams and hierarchical clustering of genes that were up-regulated (left) and down-regulated (right) in wild-type plants after RB treatment, and of genes whose expression varied between RB-treated *lox1 lox5* and *eto1-14* mutants and RB-treated wild-type plants. Three sub-clusters are shown, representing genes whose expression varied in wild-type and *lox1 lox5* (left), in wild-type and *eto1-14* (right), or in wild-type, *lox1 lox5* and *eto1-14* simultaneously (middle). Colours indicate fold change values of differentially expressed genes on a scale of −3.0 to 3.0. A complete list of genes is given in Table S1.

mutant, which generates singlet oxygen (op den Camp *et al.*, 2003; Gadjev *et al.*, 2006), revealed that, despite important experimental differences (light-/dark-grown seedlings and non-localized production of singlet oxygen after RB treatment versus chloroplastic production of singlet oxygen in leaves of mature *flu* plants after a dark/light shift), almost 60% of the genes that showed increased expression in the *flu* mutant were also induced in wild-type plants after RB (Figure S8). Moreover, in our microarray, we found induction of genes that are known to be selectively up-regulated by singlet oxygen, but not by superoxide or hydrogen peroxide, indicating that the singlet oxygen generated by RB had specific signal activity rather than cytotoxic activity (op den Camp *et al.*, 2003; Gadjev *et al.*, 2006; Przybyla *et al.*, 2008).

Gene expression after RB treatment varied greatly in *lox1 lox5* and *eto1-14* mutants compared with wild-type seedlings. Of the RB up-regulated genes in wild-type plants, 51 and 12% showed at least twofold reduced expression in the *lox1 lox5* and *eto1-14* mutants, respectively, and only 22 genes showed increased expression in *eto1-14* plants. Similar figures were found for the genes down-regulated after RB treatment in wild-type plants, as 20 and 9% of these genes showed higher expression (two-fold or more) in RB-responding *lox1 lox5* and *eto1-14* mutants, respectively, while 3 and 2% of the genes showed reduced expression in *lox1 lox5* and *eto1-14* mutants in comparison to wild-type plants. We found that expression of 9% of the RB-responsive genes in wild-type plants (RB-induced and RB-repressed) was simultaneously modified in *lox1 lox5* and *eto1-14* mutants (Figure 4 and Table S2). Moreover, 74% of the genes that showed aletered expression in the *eto1-14* mutant compared to wild-type plants were also altered in *lox1 lox5* mutants, indicating that ET preferentially affects a subset of the transcriptional response mediated by 9-LOX activity.

Further examination of our microarrays showed transcriptional changes in *lox1 lox5* and *eto1-14* mutants that were not detected in RB-responding wild-type plants (Table S3). Thus, the expression changes of 1439 genes in *lox1 lox5* and 140 genes in *eto1-14* were unique to these mutants. Furthermore, an additional group of 203 genes was found to show altered expression in both *lox1 lox5* and *eto1-14* mutants, but not in RB-treated wild-type plants.

The possible biological function of the RB-responding genes was assessed by Gene Ontology (GO) term enrichment (Table S4). Among RB up-regulated genes, we found a significant overrepresentation of genes associated with various types of stress, regulation of gene expression, the metabolism of oxylipins and ET signalling. Terms associated with cell-wall organization and lipid metabolism were overrepresented in the pool of RB down-regulated genes in wild-type plants. Moreover, among genes whose expression varied in mutants but not in wild-type plants, we found an enrichment of terms associated with abiotic stress and photosynthesis among RB up-regulated *lox1 lox5* genes, and with amino acid, phenylpropanoid and flavonoid metabolic processes among *eto1-14* up-regulated genes.

Lack of 9-LOX activity and constitutive ET production enhance the susceptibility of plants to singlet oxygen

The altered gene expression in *lox1 lox5* and *eto1-14* mutants after RB production was indicative of an impaired response to singlet oxygen. The significance of this variation was evaluated by measuring ion leakage (as an indicator of cellular damage) in the leaves of adult plants subjected to RB treatment, as well as by examining the phenotype of seedlings when grown in the presence of RB. These analyses revealed an increase in ion leakage in *lox1 lox5* and *eto1-14* mutants compared to wild-type plants (Figure 5). Thus, the approximately 1.5-fold increase in ion leakage measured in wild-type plants 24 h after RB treatment increased

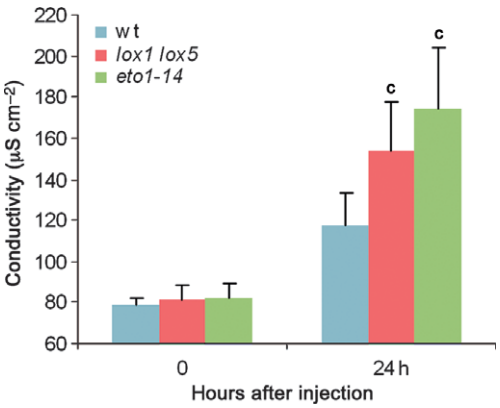

Figure 5. Measurement of ion leakage.
Conductivity measured in full-grown leaves of wild-type, *lox1 lox5* and *eto1-14* plants after injection with Rose Bengal (2 μM). Values are means and standard errors from three independent experiments are shown. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's *t* test: $0.01 < P < 0.05$).

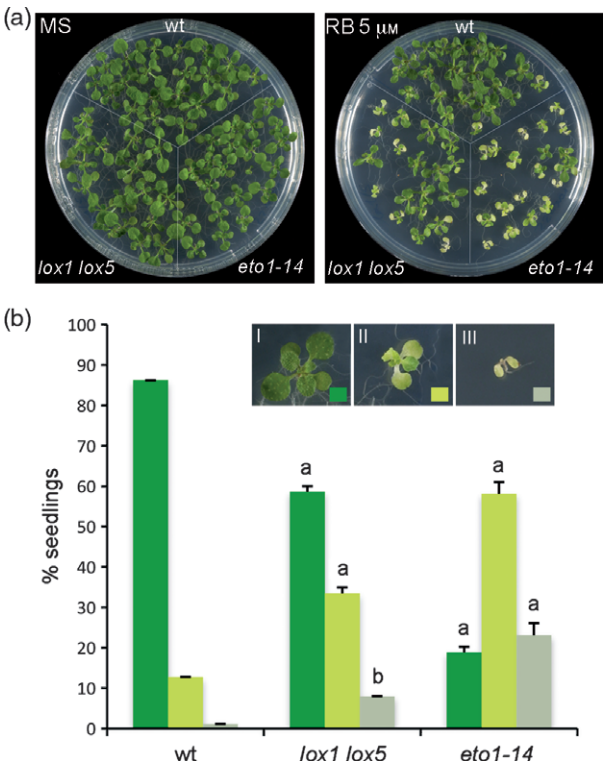

Figure 6. Analyses of phenotypic alterations in wild-type, *lox1 lox5* and *eto1-14* plants grown in RB-containing medium.
(a) Phenotype of seedlings grown for 15 days in MS medium (left) or in MS medium containing RB (5 μM) (right).
(b) Phenotypic alterations were scored on a three-point scale (I, II and III), according to the severity of the symptoms. The percentages of seedlings showing these phenotypes after 15 days of growth in RB-containing medium are shown. Values are means and standard errors from three independent experiments. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's *t* test: $^{a}P < 0.001$, $^{b}0.001 < P < 0.01$).

approximately twofold above basal levels in *lox1 lox5* and *eto1-14* mutants. Furthermore, we observed significant phenotypic variations in 15-day-old seedlings grown on medium containing 5 μM RB (Figure 6a). Rating of the phenotypic symptoms on a three-point scale (I, II and III), according to their severity, revealed a high proportion of wild-type seedlings (approximately 85%) with type I phenotype (only green leaves), compared with approximately 60 and 20% of seedlings in *lox1 lox5* and *eto1-14* mutants, respectively (Figure 6b), whereas the remaining seedlings showed type II (with green and yellow leaves) and type III symptoms (only yellow leaves). These results reveal the enhanced susceptibility of these mutants to singlet oxygen. The fact that RB caused stronger damage in *eto1-14* than in *lox1 lox5* seedlings contrasted with the reduced number of transcriptional changes found in *eto1-14*, suggesting that the qualitative differences distinguishing the transcriptional response of *lox1 lox5* and *eto1-14* to RB are critical in modulating their adaptation to oxidative stress.

Lack of 9-LOX activity and constitutive ET production enhance bacterial susceptibility and malondialdehyde accumulation

Given the attenuated response of *lox1 lox5* and *eto1-14* to singlet oxygen, and that, as described above, the genes induced by singlet oxygen in wild-type plants were enriched in responses to pathogens, we tested whether the defence potential of these mutants was diminished and whether this correlated with altered ROS regulation. Analyses of the response to the biotrophic bacterial strains *Pst* DC3000 *avrRpm1* (avirulent) and *Pst* DC3000 (virulent) revealed higher bacterial growth rates in *lox1 lox5* and *eto1-14* mutants than in wild-type controls (Figure 7a). The growth of *Pst* DC3000 *avrRpm1* did not show a significant variation in *lox1 lox5*, but increased sevenfold in *eto1-14* relative to wild-type plants. Moreover, the growth of *Pst* DC3000 increased ten- and sevenfold in *lox1 lox5* and *eto1-14*, respectively, compared with controls. Further analyses of 9-HOT-responsive gene expression revealed that, compared with wild-type plants, enhanced bacterial growth was accompanied by delayed and reduced expression, which was more apparent in the case of the virulent bacteria (Figure 7b). Analysis of the symptoms caused by bacterial inoculation revealed a slightly decreased necrosis (visible by staining with trypan blue) and increased H_2O_2 accumulation (as concluded from the strong brown coloration observed by staining with 3,3′-diaminobenzidine tetrachloride) during both interactions in *lox1 lox5* and *eto1-14* mutants (Figure 7c). Finally, we measured the accumulation of malondialdehyde (MDA), a product of uncontrolled lipid oxidation (Farmer and Davoine, 2007). As shown in Figure 8(a), there was a small but

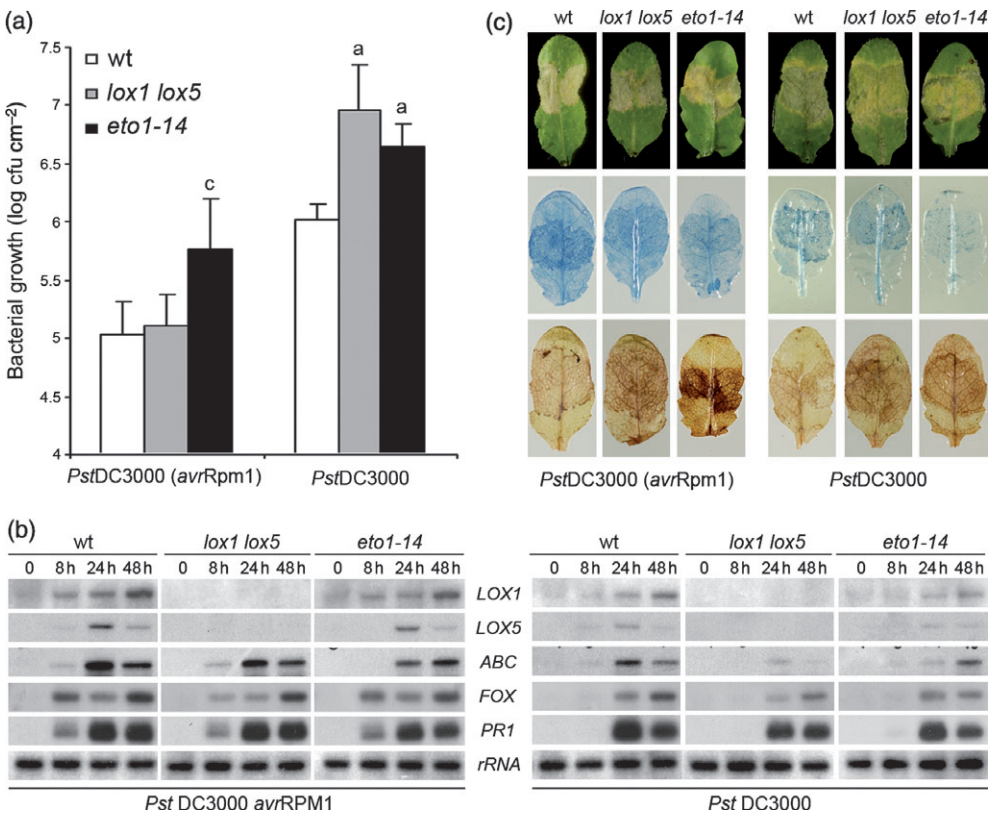

Figure 7. Response of *lox1 lox5* and *eto1-14* mutants to bacterial inoculation.
(a) Growth of *Pst* DC3000 *avrRpm1* and *Pst* DC3000 in plants 4 days after bacterial infiltration (10^5 cfu ml^{-1}). Values are means and standard errors obtained in three independent experiments. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's *t* test: $^b0.001 < P < 0.01$, $^c0.01 < P < 0.05$).
(b) Analyses of gene expression after bacterial inoculation (10^6 cfu ml^{-1}). Blots were hybridized to riboprobes for *LOX1*, *LOX5*, *ABC*, *FOX* and *PR1* genes. Hybridization against an rRNA radioactive probe was used as a loading control.
(c) Representative examples of lesions that developed in the leaves after bacterial infiltration (upper panels). Staining with trypan blue (middle panels) and 3,3′-diaminobenzidine tetrachloride (lower panels) was performed 24 and 48 h, respectively, after *Pst* DC3000 *avrRpm1* and *Pst* DC3000 inoculation.

significant increase in MDA in response to *Pst* DC3000 *avrRpm1* in *lox1 lox5* and *eto1-14* plants. A twofold increase above the levels in wild-type plants was measured in *lox1 lox5* mutants at 4 and 8 h post-inoculation, decreasing by 24 h to the levels found in wild-type plants. The MDA level in *eto1-14* mutants at 8 h post-inoculation was 1.5 times that in wild-type plants, and remained at similar levels 24 h after inoculation. Analyses of the response to *Pst* DC3000 revealed 1.8- and 1.5-fold increases in the levels of MDA in *lox1 lox5* and *eto1-14* plants, respectively, compared with wild-type plants at 8 h after bacterial infiltration.

DISCUSSION

We have previously shown that the 9-LOX product 9-HOT regulates stress responses that commonly occur during the defence of plants to pathogen infection (Vellosillo *et al.*, 2007). To obtain further insight into the molecular mechanisms involved in 9-HOT signalling, as well the role of the 9-LOX oxylipin pathway, we used a genetic approach to generate a double *lox1 lox5* Arabidopsis mutant lacking 9-LOX activity, as well as 9-HOT signalling mutants (*non-responding to oxylipins*) with an impaired response to 9-HOT. The *noxy6* and *noxy22* mutants were selected based on their insensitivity to 9-HOT (Figures S1 and S2). Identification of the *noxy6* and *noxy22* mutations as new *ctr1* (*ctr1-15*) and *eto1* (*eto1-14*) alleles, respectively, strongly indicated that enhanced ET production negatively regulates 9-HOT signalling (Figures S3 and S4). In support of this finding, we observed that application of the ET precursor ACC, in combination with 9-HOT, provoked a clear reduction of the responses to 9-HOT in wild-type plants but not in the ethylene-insensitive mutant *ein2-5* (Figure 1). Moreover, gene induction by 9-HOT reached higher levels in the *ein2-5* mutant than in wild-type plants (Figure S5), confirming a negative action of ET on the 9-HOT signalling pathway.

In addition to the inhibitory effect of ET on 9-HOT signalling, a reciprocal interference was also found, indicating that the antagonistic action of ET and 9-HOT is bi-directional. Results showing that the presence of 9-HOT reduced the ACC-induced accumulation of EIN3–GFP in

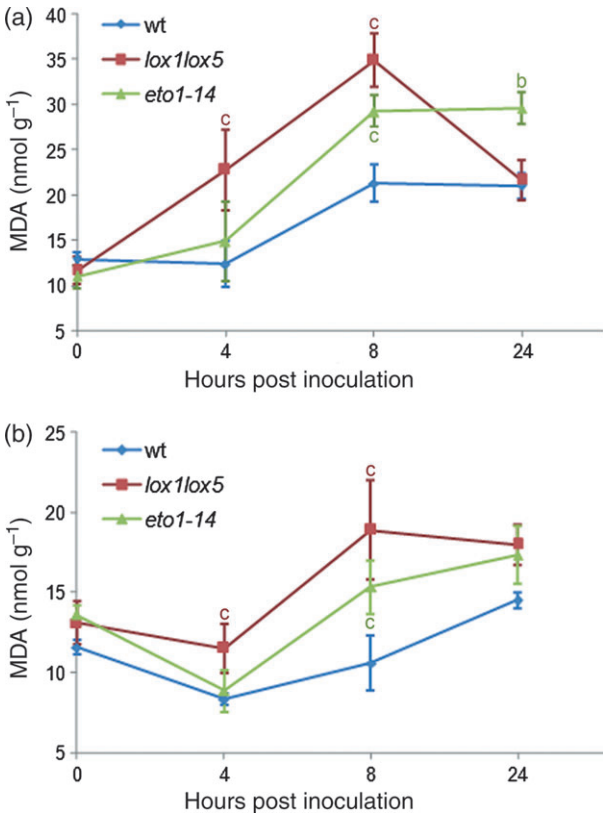

Figure 8. Analyses of MDA accumulation.
(a) Total (free and bound) MDA levels measured by GC-MS in the leaves of wild-type, *lox1 lox5* and *eto1-14* at various intervals after *Pst* DC3000 *avrRpm1* infiltration (10^7 cfu ml^{-1}).
(b) Total (free and bound) MDA levels measured by GC-MS in the leaves of wild-type, *lox1 lox5* and *eto1-14* at different intervals after *Pst* DC3000 infiltration (10^7 cfu ml^{-1}).
Values are means and standard errors from three independent experiments. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's *t* test: $^b0.001 < P < 0.01$, $^c0.01 < P < 0.05$).

35S:EIN3-GFP transgenic plants, as well as of GUS activity in roots of *EBS:GUS* plants (Figure 2), demonstrated that 9-HOT interferes with activation of the ET pathway. Similar interference with the ET pathway was found with 9-KOT, which, like 9-HOT, induces root waving, whereas the opposite was observed with JA or 9-Oxo-C_9, which induce root growth arrest through a 9-HOT-independent signalling pathway (Figure 2 and Figure S1). The positive interaction of the JA and ET pathways was in accordance with previous results showing synergistic interaction of these hormones during the response of plants to necrotrophic pathogens (Glazebrook, 2005). On the other hand, the fact that the interaction between JA and ET differed from that of 9-HOT and ET, supported our previous data suggesting signalling diversification of these two oxylipins, JA and 9-HOT. Moreover, the fact that the action of 9-HOT and 9-KOT (9-LOX derivatives) on ET signalling differed from that of 9-Oxo-C_9 (which is also produced by the 9-LOX oxylipin pathway), supported our early results suggesting functional specialization of oxylipins according to their molecular structure rather than the specific biochemical pathway (9-LOX, 13-LOX or α-DOX) mediating their production (Vellosillo *et al.*, 2007).

The fact that application of Trolox, a tocopherol analogue with lipophilic anti-oxidant activity (Girotti, 1998), impaired the effects of 9-HOT (Figure 3), suggested involvement of oxidative stress and lipid peroxidation in the 9-HOT response. Moreover, the fact that 9-HOT enhanced ROS production was indicative of a model in which 9-HOT potentiates activation of the 9-LOX pathway in a positive feedback loop. Additionally, these results suggest participation of the 9-LOX oxylipin pathway in controlling oxidative stress and lipid peroxidation. This is supported by the results of transcriptional analyses of wild-type plants, the *lox1 lox5* mutant (lacking 9-LOX activity) and the *eto1-14* mutant (disrupting 9-HOT signalling) after application of RB, a generator of singlet oxygen with strong lipid peroxidation potential (Przybyla *et al.*, 2008; Triantaphylidès and Havaux, 2009). The fact that, of the 1832 genes responding to RB in wild-type plants, 51 and 12% showed altered expression in *lox1 lox5* and *eto1-14*, respectively, demonstrated involvement of the 9-LOX and ET pathways in the response of plants to singlet oxygen (Figure 4). In addition, the higher levels of ion leakage (an indicator of cellular damage) and increased chlorosis in tissues of RB-treated *lox1 lox5* and *eto1-14* mutants than in RB-treated wild-type plants (Figures 5 and 6) confirmed that both mutants are defective in signalling an appropriate response to singlet oxygen, and revealed their diminished ability to control this type of oxidative damage. As reported previously (Sattler *et al.*, 2004), tocopherols (vitamin E) quench singlet oxygen in a highly efficient manner, and their activity to limit lipid peroxidation is essential for germination and early plant growth. Therefore, the enhanced damage in RB-treated *lox1 lox5* and *eto1-14* mutants relative to control plants may result, at least in part, from failure to control lipid peroxidation after application of singlet oxygen.

Further studies in adult plants revealed the participation of 9-LOX and ET in the defence of plants against virulent bacteria (Figure 7). Differing actions of the 9-LOX and ET pathways in this response were deduced from the enhanced susceptibility to *Pst* DC3000 in *lox1 lox5* and *eto1-14* mutants compared with wild-type plants. The fact that the double *lox1 lox5* mutant supported approximately tenfold increased bacterial growth compared with the approximately fivefold increase found in the Arabidopsis *lox1* mutant (Hwang and Hwang, 2010, and our unpublished data, Centro Nacional de Biotecnología, Madrid, Spain) indicates the positive contribution of the two 9-LOX genes, *LOX1* and *LOX5*, to plant defence. In line with these results, the enhanced suscepti-

bility of the *eto1-14* plants could be, at least in part, a consequence of the antagonistic action of ET on 9-HOT signalling, and is in agreement with a study by Chen *et al.* (2009) showing that constitutive ET production decreased defence gene expression. In addition, recent reports revealed a positive role for ET as a regulator of pathogen-associated molecular pattern-triggered immunity (Boutrot *et al.*, 2010; Mersmann *et al.*, 2010), indicating that ET may exert different actions in response to pathogens that are operative at various layers of defence.

The results showing increased H_2O_2 accumulation in *lox1 lox5* and *eto1-14* mutants after bacterial inoculation compared with wild-type plants (Figure 7c) are indicative of an alteration in the regulation of ROS. Moreover, the enhanced accumulation of MDA, a product of lipid peroxidation, in *lox1 lox5* and *eto1-14* mutants after bacterial infection (Figure 8) is probably a reflection of a failure in signalling responses to highly reactive oxygen species, such as hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$). The fact that the *lox1 lox5* and *eto1-14* mutations may affect distinct types of ROS could be indicative of a broad regulatory role of the 9-LOX oxylipin and ET pathways on various ROS signalling pathways. Alternatively, the alterations observed could be a consequence of the interaction between distinct ROS signalling pathways as has been shown by Laloi *et al.* (2007). Independently of this, the data described suggest actions of the 9-LOX and ET pathways in the control of oxidative stress during the response to pathogen infection, in which the production of ROS must be tightly regulated to achieve full resistance and plant survival.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana wild-type, transgenic lines *35S:EIN3-GFP* and *EBS:GUS*, and *ein2-5* mutants were derived from ecotype Columbia (Col-0). The homozygous double insertion mutant *lox1 lox5* was generated by crossing individual mutants and identified by PCR as described in Vellosillo *et al.* (2007). For *in vitro* analyses, vernalized seeds were grown in Petri dishes containing Murashige & Skoog (MS) medium, pH 6.0, 1.5% w/v sucrose and 1.5 or 0.8% w/v agar (Bacto Agar; Becton-Dickinson, http://www.bd.com) for vertical or horizontal plates, respectively. Growth conditions were 16 h light/8 h dark at 22°C, with 250 μE m^{-2} sec^{-1} fluorescent illumination. Specific compounds were added to molten medium (50°C) at the indicated concentrations. Freshly prepared plates were always used to avoid product breakdown or instability. Phenotypes were observed 6 days after seed germination in approximately 15 independent seedlings (see Figure S9 as an example), and in at least three independent experiments. For *in planta* analyses, vernalized seeds were sown on soil, and grown in a growth chamber at 22°C and 70% relative humidity under a 14 h light/10 h dark photoperiod with 250 μE m^{-2} sec^{-1} fluorescent illumination.

Preparation of oxylipins

9(*S*)-hydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-HOT) was prepared by stirring linolenic acid (120 mg) at 23°C with *Solanum lycopersicum* whole homogenate under an atmosphere of oxygen, essentially as described by Matthew *et al.* (1977). The product was subjected to open-column silicic acid chromatography to provide >95% pure 9(*S*)-hydroperoxyoctadecadienoic acid (HPOD) (70 mg). Treatment with sodium borohydride (100 mg) in methanol (10 ml) at 0°C for 30 min followed by preparative straight-phase HPLC using 2-propanol/hexane/acetic acid (2.2:97.8:0.005 v/v/v) as the mobile phase produced >99% pure 9-HOT as a colorless oil (44 mg; yield 35% from linolenic acid).

9-keto-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-KOT) was prepared by treating 9(*S*)-HPOD (125 mg) at 0°C for 5 min with a mixture of acetic anhydride (3 ml) and pyridine (2.4 ml). Water (1.5 ml) was added, and the mixture was stirred at 23°C for 60 min. Purification by straight-phase HPLC using 2-propanol/hexane/acetic acid (1.1:98.9:0.005 v/v/v) as the mobile phase afforded >97% pure 9-KOT as a white solid (91 mg; yield 77%).

9-oxononanoic acid (9-Oxo-C_9) was prepared by stirring 9,10-dihydroxyoctadecanoic acid (2 g) in acetone (100 ml), water (25 ml) and acetic acid (5 ml) with sodium periodate (4 g) at 23°C under argon for 90 min. The product was subjected to silica gel chromatography, followed by crystallization from hexane at 4°C, producing >98% pure 9-Oxo-C_9 as a white solid (0.41 g; yield 38%).

Assay of 9-LOX activity

Roots (0.2 g) or liquid nitrogen-powderized leaves (0.5 g) were homogenized using an Ultra-Turrax disperser (IKA-Werke, http://www.ika.net) at 0°C in 3 ml of 0.1 M potassium phosphate buffer, pH 6.7, containing 300 μM α-linolenic acid. The homogenates were stirred at 23°C for 20 min, and subsequently treated with 6 ml methanol containing 75 μg butylated hydroxytoluene anti-oxidant and 47.8 nmol of [17,17,18,18-$^2H_5$]-9-HOT as an internal standard. After centrifugation at 700 ***g***, oxylipins were isolated by extraction with diethyl ether. The products were derivatized by methyl esterification and trimethylsilylation and analysed by GC-MS as previously described (Hamberg *et al.*, 2003). The instrument was operated in scan mode for profiling of oxylipins and in selected ion monitoring mode for determination of levels of 9-HOT.

Histochemistry

Detection of callose and superoxide production was performed as described by Vellosillo *et al.* (2007). Production of H_2O_2 was visualized in detached leaves stained with 3,3′-diaminobenzidine tetrachloride (Sigma, http://www.sigmaaldrich.com/) as described by Thordal-Christensen *et al.* (1997) and Moreno *et al.* (2005). For cell death analyses, leaves were stained as described by Hamberg *et al.* (2003). *In situ* localization of GUS activity was performed as described by Malamy and Benfey (1997).

Protein extraction and Western blot

Six-day-old seedlings of *35S:EIN3-GFP* transgenic plants were transferred to liquid MS medium and subjected after 24 h to ACC (2 μM) treatment alone or in combination with 25 μM of the oxylipins (9-HOT, 9-KOT, JA or 9-Oxo-C_9). Protein extracts were prepared by grinding seedlings to a fine powder in liquid nitrogen, and extracted using buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM PMSF and 1× complete protease inhibitor cocktail (Roche, http://www.roche.com). Supernatants were obtained by centrifugation for 10 min at 3000 ***g***, separated by 10% SDS–PAGE, and transferred to nitrocellulose membranes by electroblotting. Immunoblot assays were performed as described by Sanz *et al.* (1998) using anti-Arabidopsis α-EIN3 antibody (Guo and Ecker,

2003) or anti-Arabidopsis α-RPT5 (19S regulator ATPase subunit Rpt5) antibody (Kwok *et al.*, 1999) as a loading control.

Plant treatments and gene expression analyses

For chemical treatment, RNA was prepared from 12-day-old seedlings grown on vertical MS-containing plates. Seedlings were covered with liquid MS medium (used as a control), or with liquid MS containing 9-HOT (25 μM) or RB (10 μM). Two square plates with 30–40 seedlings per plate were pooled for each time point examined. For studies of gene expression after bacterial inoculation, the abaxial side of rosette leaves of 4-week-old plants were injected with a bacterial suspension (10^6 cfu ml^{-1}). In all cases, tissues were frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was isolated as described by Logemann *et al.* (1987). RNA gel blots were performed as described by Sambrook and Russell (2001), transferred to Hybond N membranes (GE Healthcare, http://www.gehealthcare.com), and hybridized to single-stranded riboprobes according to standard procedures. In all cases, gene expression was analysed in at least three independent experiments. Radioactive probes were prepared for *LOX1* (At1g55020), *LOX5* (At3g22400), *POX* (At5g22140), *ABC* (At1g15520), *FOX* (At1g26380) and *PR1* (At2g14160). The amount of loaded RNA was verified by addition of ethidium bromide to the samples and photography under UV light after electrophoresis, followed by hybridization to an rRNA as described by Vellosillo *et al.* (2007).

Microarray hybridization and analysis

For microarray analyses, RNA was extracted 3 h after treatment and purified using an RNeasy mini kit (Qiagen, http://www.qiagen.com/). RNA was quantified using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technology Inc., http://www.nanodrop.com), and its quality was assessed using an Agilent 2100 bioanalyser (Agilent Technologies, http://www.agilent.com). RNA from four independent biological replicates was prepared and independently hybridized in three independent experiments using slides of Agilent Arabidopsis oligo microarrays 4 × 44K (reference 021169). Differential expression comparisons were performed as follows: (i) MS-treated wild-type Arabidopsis seedlings versus wild-type seedlings treated with RB-containing MS medium (10 μM), (ii) RB-treated wild-type seedlings versus RB-treated *lox1 lox5* mutant seedlings, and (iii) RB-treated wild-type seedlings versus RB-treated *eto1-14* mutant seedlings. Details of hybridization and washing of microarrays are given in Appendix S1. Raw and normalized data were deposited in the Array Express data library (http://www.ebi.ac.uk/arrayexpress/) under accession numbers E-MEXP-3009, E-MEXP-3010 and E-MEXP-3011. Significantly up-regulated and down-regulated transcripts obtained for each comparison (with a false discovery rate of 5%) are listed in Tables S1–S4. The fold change representing the differential expression is listed for each probe.

Analysis of gene ontology functional term enrichment

We used the FatiGO application (Al-Shahrour *et al.*, 2004) contained in the suite of web tools Babelomics version 3.2 (Al-Shahrour *et al.*, 2006). The server at the Centro de Investigación Príncipe Felipe (Valencia, Spain) was used to obtain an overview of the Gene Ontology (GO) functional term enrichment for RB-responsive genes. Hierarchical clustering of significant terms (adjusted *P* value <0.001) was performed using Multiexperiment Viewer (MeV) software version 4.3 (http://www.tm4.org) (Saeed *et al.*, 2003).

In vivo analyses of bacterial symptoms and *in vivo* growth curves

The pathogenic bacterial strains used in this study, *Pseudomonas syringae* pv. *tomato* DC3000 and the avirulent strain *Pseudomonas* DC3000 *avrRpm1*, were grown overnight at 28°C in Petri plates containing King's medium B. Bacterial inoculation and quantification of bacterial growth were performed as described by De León *et al.* (2002). Data were statistically analysed using Student's *t* test using GraphPad PRISM version 4 (http://www.graphpad.com/prism/Prism.htm). For symptoms tests, at least 20 plants were examined after bacterial infiltration (10^6 cfu ml^{-1}) in three independent experiments. Reported data are means and standard errors of the values obtained in three independent experiments.

Measurement of ion leakage

Cell damage was assayed by measuring ion leakage as described by De León *et al.* (2002). The abaxial side of leaves of 4-week-old plants were injected with 30 μl Rose Bengal solution (2 μM). Three leaves were treated per plant. Reported data are means and standard errors of the values obtained in three independent experiments. Data were statistically analysed by Student's *t* test as described above.

Analysis of malondialdehyde by GC-MS

Leaves were infiltrated with suspensions of *Pst* DC3000 *avrRpm1* (10^7 cfu ml^{-1}) or *Pst* DC3000 (10^7 cfu ml^{-1}). Leaf tissue was harvested at 4, 8 and 24 h post-inoculation, frozen in liquid nitrogen, and stored at −80°C until analysis. The level of malondialdehyde (MDA) was measured essentially as described by Cighetti *et al.* (2002), using methyl malondialdehyde as an internal standard and gas chromatography/mass spectrometry (GC-MS). Data were statistically analysed by Student's *t* test as described above. A detailed description of the extraction and quantification procedures is given in Appendix S2.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phenotypic response of *noxy6* and *noxy22* to oxylipins.

Figure S2. Response of *noxy6* and *noxy22* to 9-HOT.

Figure S3. Map-based cloning of *noxy6* and *noxy22*.

Figure S4. Phenotype of dark-grown wild-type, *ctr1-15* and *eto1-14* seedlings.

Figure S5. Expression 9-HOT-responsive genes in wild-type and *ein2-5* seedlings.

Figure S6. Phenotype of wild-type seedlings grown in the presence of redox-active compounds.
Figure S7. Scheme of genomic structures of LOX1 and LOX5 and position T-DNA insertions.
Figure S8. Comparison of transcriptional profiles from RB-treated wild-type seedlings and *flu* mutants.
Figure S9. Phenotypes of wild-type seedlings grown in vertically oriented plates.
Figure S10. Calibration curve for determination of MDA.
Table S1. Genes differentially expressed after RB treatment.
Table S2. Genes responding to RB treatment whose expression is affected by the *lox1 lox5* and *eto1-14* mutations.
Table S3. Genes whose expression changed after RB treatment in *lox1 lox5* and *eto1-14* mutants.
Table S4. GO term enrichment of RB-responsive genes.
Appendix S1. Details of microarray hybridization.
Appendix S2. Details of malondehayde determination.
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